UNIVERSITÉ DU QUÉBEC À MONTRÉAL

PROTECTIVE ROLE OF MILD THERMOTOLERANCE (40°C) AGAINST APOPTOSIS INDUCED BY OXIDATIVESTRESS

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ROLE PROTECTEUR DE LA THERMOTOLERANCE (40°C) CONTRE L'APOPTOSE INDUITE PAR LE STRESS OXIDATIF

THÈSE

PRÉSENTÉE

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To my beloved parents

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LIST DES ABREVIATIONS

AFC	Amino trifluorocoumarin
AMC	Amino methylcoumarin
AIF	Apoptosis-inducing factor
APAF-1	Apoptosis protease activating factor-1
Ac-DEVD-AMC	Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin
Ac-LEHD-AFC	Ac-Leu-Glu-His-Asp-AFC
Ac-LEVD-AFC	Ac-Leu-Glu-Val-Asp-AFC
ATF6	Activating transcription factor-6
BAX	Bcl-associated X protein
BCL-2	B cell lymphoma 2
BSA	Bovine serum albumin
BAPTA-AM	1, 2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester)
CuZnSOD	Copper-zinc superoxide dismutase
CAD	Caspase activated DNase
CARD	Caspase recruitment domain
CYT C	Cytochrome c
CHAPS	3 - [(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonic acid

DIABLO/SMAC	Direct IAP Binding Protein with Low PI/Second Mitochondria-derived Activator of Caspases
DNA	Deoxyribose nucleic acid
D-MEM	Dulbecco's modification of Eagle's medium
DISC	Death-inducing signalling complex
DTT	Dithiothreitol
Endo G	Endonuclease G
ER	Endoplasmic reticulum
ETC	Electron transport chain
ERAD	ER-associated protein degradation
eIF2α	Eukaryotic initiation factor 2 alpha
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol (2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ECL	Electrochemiluminescence
FBS	Fetal bovine serum
FCCP	*P*-trifluoromethoxy-phenyl-hydrazone
FasL	Fas ligand
Fas	Fas receptor
FADD	Fas-associated death domain
c-FLIP	Cellular FLICE inhibitory protein
FMK	Fluoromethyl ketone
FACS	Fluorescent activated cell sorter

GRP	Glucose related protein
Gpx	Glutathione peroxidise
GST-π1	Glutathione S-transferase-π1
γ-GCS	Gamma-glutamyl cysteine synthetase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HtrA2/Omi	High-temperature requirement A2 serine protease
HeLa	Human cervical carcinoma cells
H_2O_2	Hydrogen peroxide
Hsps	Heat shock proteins
IRE1	Inositol-requiring protein -1
IAPs	Inhibitor of apoptotic proteins
JNK	c-Jun N-terminal kinase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine
L-BSO	L-buthionine sulfoximine
LC3	Light chain 3
MPT	Mitochondrial permeability transition
MnSOD	Manganese superoxide dismutase
MOPS	3-(*N*-morpholino)-propane sulfonic acid
MOMP	Mitochondrial outer membrane permeabilization
NF-κB	Nuclear transcription factor κB
NaCl	Sodium chloride

NADPH	Nicotinamide adenine dinucleotide phosphate
PUFA	Poly unsaturated fatty acids
PERK	Protein kinase RNA (PKR)-like ER kinase
PARP	PolyADP-ribose polymerase
PBS	Phosphate-buffered saline
PMSF	Phenylmethylsulfonyl fluoride
PEG	Polyethylene glycol
PI	Propidium iodide
PTP	Permeability transition pore
PDI	Protein disulphide isomerise
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
RIP1	Receptor-interacting kinase 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
SOD	Superoxide dismutase
t-Bid	Truncated bid
TNF	Tumor-necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R1	Trail receptor-1

TNFR	Tumor necrosis factor receptor
TRADD	TNF-receptor-associated death domain
TRAF-2	TNF-receptor-associated factor-2
UPR	Unfold protein response
XIAP	X-linked inhibitor of apoptosis protein
Z-IETD-AFC	Z-Ile-Glu-Thr-Asp-AFC
Z-VDVAD-AFC	Z-Val-Asp-Val-Ala-Asp-AFC
Z-ATAD-FMK	Benzyloxycarbonyl-Ala-Thr-Ala-Asp-FMK

RÉSUMÉ

Une faible exposition à des stress tels qu'un choc thermique, du stress oxydatif ou des radiations peut induire une réponse adaptative qui permet aux cellules et aux organismes qu'elles constituent de continuer à fonctionner normalement lorsqu'ils sont confrontés à un stimulus négatif. Ces réponses adaptatives impliquent de nombreux changements dans l'expression de gènes et de protéines, dont l'induction de défenses cellulaires (antioxydants, protéines de choc thermique (heat shock proteins (Hsps)), etc.) qui permettront à la cellule de survivre. Si une réponse adaptative ne parvient pas à contrer les effets de l'exposition au stress, les cellules sont éliminés via des processus de mort cellulaire comme l'apoptose ou la nécrose. Une exposition préalable à des températures modérées, de l'ordre de 40°C, induit une réponse adaptative (la thermotolérance) qui permet aux cellules de résister à une agression toxique ultérieure. La thermotolérance induite par des températures modérées de l'ordre de 39-40°C (ce qui correspond physiologiquement à un état fiévreux) a été peu étudiée et est mal comprise. Les deux objectifs principaux de cette étude sont: (i) d'évaluer si l'induction d'un système de défense par une température modérée et non-létale (40°C) peut permettre de protéger la cellule contre l'activation de la cascade apoptotique par le stress oxydatif, et (ii) de comprendre les mécanismes moléculaires détaillés de l'apoptose induite par le stress oxydatif. L'apoptose est évaluée au niveau des voies de signalisation médiées par les mitochondries, des recepteurs de mort et le réticulum endoplasmique (RE) dans des cellules HeLa.

L'objectif premier est de déterminer si un choc thermique modéré peut induire un mécanisme de défense cellulaire autre que les Hsps. En effet, une thermotolérance modérée (40°C, 3h) induit une augmentation du taux de plusieurs antioxydants et protéines de stress du RE. L'expression protéique et l'activité enzymatique de la manganèse superoxyde dismutase (MnSOD) et de la catalase sont augmentées dans les cellules thermotolérantes, comparées aux contrôles (37°C). Les niveaux intracellulaires de glutathion et de γ-glutamylcysteine synthétase, l'enzyme qui synthétise le glutathion, sont aussi augmentés. Ceci peut être expliqué par l'augmentation de la production de dérivés réactifs de l'oxygène (reactive oxygen species, ROS) dans les cellules thermotolérantes. De plus, l'expression des protéines de stress du RE, PERK, p-PERK, eIF2α et p-eIF2α, est accrue dans ces cellules. Cela démontre que la thermotolérance modérée accroit les effets pro-survie de la voie PERK/eIF2α de réponse aux mauvais repliements de protéines (unfolded protein response, UPR).

Le second objectif a pour but de déterminer les mécanismes de l'apoptose induite par le stress oxydatif. Lorsqu'elles sont exposées à du peroxyde d'hydrogène (H_2O_2), les cellules entrent en apoptose via les voies des mitochondries, des recepteurs de

mort et du RE. L'activation de la cascade mitochondriale de l'apoptose implique la translocation de Bax à la mitochondrie, la dépolarisation de la membrane mitochondriale, la libération du cytochrome c, l'activation des caspases -9 et -3 et la condensation de la chromatine du noyau. De plus, H_2O_2 cause l'activation de p53 et l'induction de sa protéine cible PUMA, ainsi que l'apoptose caspase-indépendante impliquant le facteur d'induction de l'apoptose (AIF). Ces évènements sont tous inhibés dans les cellules thermotolérantes, ce qui suggère qu'une exposition préalable à des températures modérées et physiologiques peut protéger les cellules contre l'apoptose mitochondriale déclenchée par le stress oxydatif, qu'elle soit caspase-dépendante ou -indépendante.

De plus, H_2O_2 active la voie d'apoptose du Fas recepteur de mort par l'induction du ligand FasL, le recrutement de la protéine FADD à la membrane plasmique et l'activation des caspases -8 et -2. Ceci mène au clivage de Bid et à la voie mitochondriale de l'apoptose. Tous ces évènements sont diminués dans les cellules thermotolérantes, ce qui démontre une fois de plus le rôle anti-apoptotique de cette réponse adaptative. L'induction de FasL, l'activation des caspases -8 et -2 et l'apoptose sont inhibées par pifithrin-α, un inhibiteur de p53, ce qui suggère que p53 agit en amont dans l'activation de la voie des death receptors par H_2O_2.

Une exposition courte (15 min) des cellules au H_2O_2 donne lieu à l'activation de l'UPR, comme le confirment l'augmentation de l'expression de p-PERK, p-eIF2α, p-IRE1α et du clivage de ATF6. Une exposition plus longue (1-3 h) au H_2O_2 induit l'apoptose liée au RE, durant laquelle l'expression de CHOP ainsi que l'activité enzymatique de la calpaïne et des caspase-7, -4, -12 et -3 augmentent. Tous ces évènements pro-apoptotiques sont diminués dans les cellules thermotolérantes. Il a été montré que le calcium, la calpaïne et la caspase-7 agissent en amont de l'activation de l'apoptose liée au RE par H_2O_2. De plus, la réponse adaptative (UPR) prédomine lors d'expositions courtes au H_2O_2 (stress léger) tandis que lors d'expositions plus longues (stress sévère), c'est l'apoptose liée au RE qui est la plus fréquente.

En conclusion, cette étude permet d'ajouter aux connaissances sur l'effet anti-apoptotique et protecteur de la réponse adaptative induite par un stress modéré, tel une fièvre, sur le stress oxydatif. Cette étude améliore également notre compréhension de l'activation de la cascade apoptotique par le pro-oxydant H_2O_2, cascade qui a d'importantes répercussions sur la santé humaine si l'on considère le rôle des ROS dans les pathologies majeures que sont le cancer, le diabète, les maladies cardiovasculaires ou encore les maladies neurodégénératives.

ABSTRACT

Low dose exposure to stresses such as heat shock, oxidative stress and radiation can induce adaptive responses, which allow cells and organisms to continue normal function in the face of adverse stimuli. Adaptive responses involve multiple changes in gene and protein expression, including induction of cellular defences (antioxidants, heat shock proteins (Hsps), etc.) to enable the cell to survive. If an adaptive response cannot counteract an adverse stress exposure, then cells are eliminated by death processes such as apoptosis or necrosis. Pre-exposure to mild temperatures such as 40°C induces an adaptive response (thermotolerance), whereby cells resist subsequent exposure to a toxic insult. The phenomenon of thermotolerance induced at moderate temperatures such as 39-40°C, within the physiological fever range, has received little attention and is not well understood. The two main objectives of this study are: (i) To evaluate whether induction of defense systems by a mild nonlethal temperature (40°C) can afford cellular protection against activation of the apoptotic cascade by oxidative stress, and (ii) To understand the detailed molecular mechanisms of apoptosis induced by oxidative stress. Apoptosis is evaluated at the levels of signalling pathways mediated by mitochondria, death-receptors and the endoplasmic reticulum (ER) in HeLa cells.

The first objective determines whether mild heat shock can induce other cellular defences besides Hsps. Indeed, mild thermotolerance (40°C, 3h) induced an increase in levels of several antioxidants and ER stress proteins. The protein expression pattern and enzymatic activity of manganese superoxide dismutase (MnSOD) and catalase were increased in thermotolerant cells, compared to controls (37°C). There were also increases in levels of intracellular glutathione and γ-glutamylcysteine synthetase, the glutathione synthesis enzyme, in these cells. This could be explained by increased generation of reactive oxygen species (ROS) in thermotolerant cells. Furthermore, expression of the ER stress proteins PERK, p-PERK, eIF2α and p-eIF2α was increased in thermotolerant cells. This shows that mild thermotolerance enhanced the pro-survival effects of the PERK/eIF2α branch of the unfolded protein response (UPR).

The second objective was to determine the mechanisms of apoptosis induced by oxidative stress. When exposed to hydrogen peroxide (H_2O_2), cells underwent apoptosis through the mitochondrial, death receptor and ER pathways. Activation of the mitochondrial cascade of apoptosis entailed translocation of Bax to mitochondria, mitochondrial membrane depolarisation, cytochrome c release, activation of caspases-9 and -3, and nuclear chromatin condensation. In addition, H_2O_2 caused activation of p53 and induction of its target protein PUMA, as well as caspase-independent apoptosis involving apoptosis-inducing factor (AIF). These events were

all inhibited in thermotolerant cells, which suggests that pre-exposure to mild physiological temperatures can protect cells against both caspase-dependent and caspase-independent mitochondrial apoptosis triggered by oxidative stress.

Moreover, H_2O_2 activated the Fas death receptor pathway of apoptosis, which was evident by induction of death ligand FasL, recruitment of adaptor protein Fas-associated death domain (FADD) to the plasma membrane, and activation of caspases-8 and -2. This led to activation of the cross-talk pathway mediated by Bid cleavage, and activation of the mitochondrial apoptotic pathway. All of these events were diminished in mild thermotolerant cells, once again demonstrating the antiapoptotic role of this adaptive response. FasL induction, activation of caspases-8 and -2 and apoptosis were inhibited by p53 inhibitor pifithrin-α, implicating p53 as an upstream factor in activation of the death receptor pathway by H_2O_2.

A short exposure (15 min) of cells to H_2O_2 led to activation of the UPR, revealed by increased expression of p-PERK, p-eIF2α, p-IRE1α, and ATF6 cleavage. Longer exposure (1-3 h) to H_2O_2 induced ER-mediated apoptosis, whereby expression of CHOP increased, as well as activity of calpain, caspase-7, -4, -12 and -3. All of these pro-apoptotic events were diminished in thermotolerant cells. Calcium, calpain and caspase-7 were shown to be upstream factors in the activation of ER-mediated apoptosis by H_2O_2. Moreover, the adaptive response (UPR) dominates at shorter exposure times to H_2O_2 (milder stress), whereas ER-mediated apoptosis occurs at longer exposure times (more severe stress).

In conclusion, this study advances knowledge about the protective anti-apoptotic effect of the adaptive response induced by exposure to mild stresses, such as fever temperatures, against oxidative stress. Furthermore, this study improves understanding about activation of the apoptotic cascade by the pro-oxidant H_2O_2, which has important repercussions for human health, given the role of ROS in major physiopathologies such as cancer, diabetes, cardiovascular diseases and neurodegenerative disorders.

CHAPTER I

INTRODUCTION

1.1 OXIDATIVE STRESS

Oxygen plays a major role in the life of aerobic organisms. It is used as a terminal electron acceptor during the production of ATP in the mitochondria by a process known as oxidative phosphorylation. This process results in the generation of both reactive oxygen and nitrogen species (ROS, RNS). The majority of ROS are products of the mitochondrial respiratory chain. The three major types of ROS include the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$). In most cases, $O_2^{\bullet -}$ is the first ROS species generated in living cells. The one-electron reduction of molecular oxygen produces a relatively stable intermediate, the superoxide anion (Figure 1.1) (Orrenius et al, 2007). Apart from the mitochondrial respiratory chain, other sources of $O_2^{\bullet -}$ include enzymes such as cytochrome P450 in the endoplasmic reticulum (ER), lipooxygenases, cyclooxygenases, xanthine oxidase and NADPH oxidase.

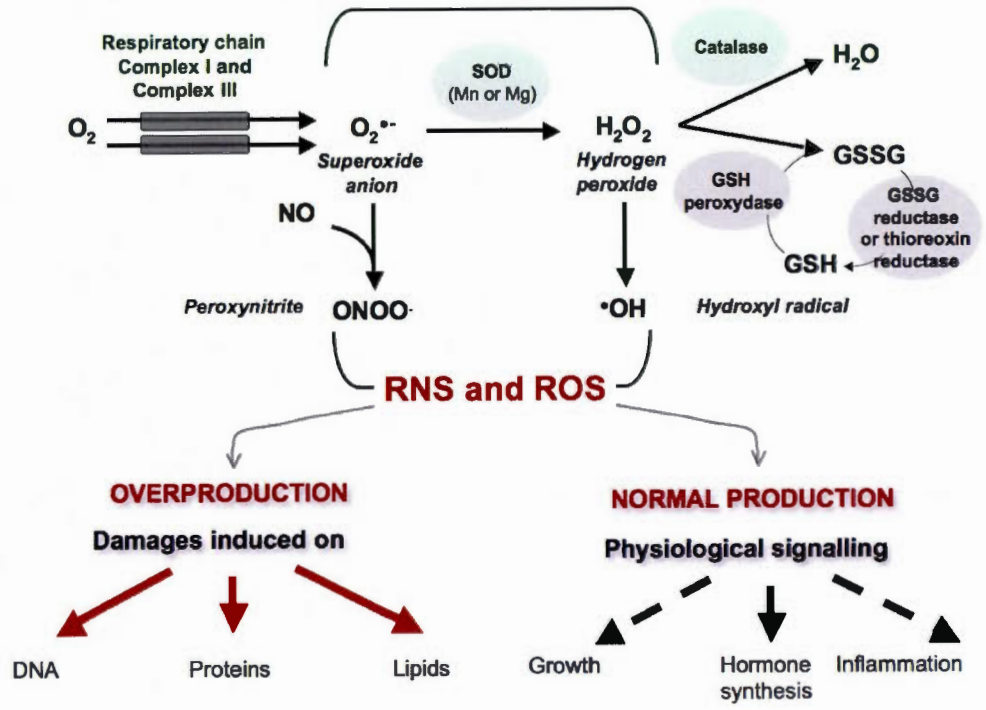


Figure 1.1 The mitochondrial formation of ROS and RNS. Figure adapted from Bellance et al, 2009 (see text for details).

The superoxide anion acts as a major precursor for the generation of various other reactive pro-oxidants, where dismutation of superoxide anion by the mitochondrial manganese-superoxide dismutase (MnSOD) produces hydrogen peroxide.

$$2\,O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Under stress conditions, an excess of superoxide releases "free iron" from iron-containing molecules (Liochev et al, 1994). The released iron (Fe^{2+}) can participate in the Fenton reaction. Generation of the hydroxyl radical by the Fenton reaction takes place in two steps:

$$(1)\ Fe\ (III) + O_2^{\bullet-} \longrightarrow Fe\ (II) + O_2$$

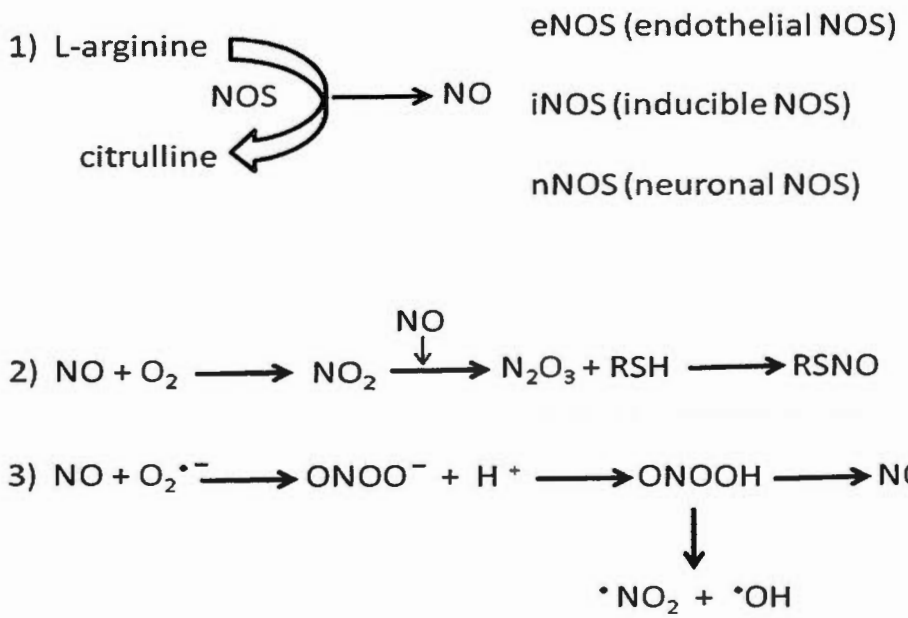
$$(2)\ Fe\ (II)\ + H_2O_2 \longrightarrow Fe\ (III)\ + OH^{\bullet} + OH^{-}$$

Thus, under stress conditions, $O_2^{\bullet -}$ acts as an oxidant of iron-sulfur [4Fe-4S] cluster-containing enzymes and facilitates $OH^{\bullet}$ production from H_2O_2 by making Fe^{2+} available for the Fenton reaction.

The highly reactive $OH^{\bullet}$ radical can also be generated by the metal catalysed Haber-Weiss reaction:

$$H_2O_2 + O_2^{\bullet -} \xrightarrow{Fe\ II/Fe\ III} O_2 + OH^{\bullet} + OH^{-}$$

Reactive nitrogen species (RNS) include •NO and their related species such as peroxy nitrite anion ($ONOO^-$). Nitric oxide is a reactive and transient radical molecule that can diffuse across the cell membrane. It is generated from guanido nitrogen of L-arginine by three distinct isoforms of nitric oxide synthase (NOS) in the cells (Pacher et al, 2007). $O_2^{\bullet -}$ undergoes a reaction with •NO to produce $ONOO^-$ (Figure 1.1). Peroxynitrite is relatively long-lived, stable molecule, which has an ability to reach critical targets of cells, as it is membrane permeable (Glebska and Koppenol, 2003). •NO related species in mitochondria including ONOO-, NO_2, N_2O_3 and S-nitrosothiols (from •NO and thiol reaction) cause slow, nonselective, weak, but irreversible or slowly reversible inhibition of many mitochondrial components including proteins, lipids and mt-DNA. Moreover, the inhibition observed in most cells in response to •NO or expression of inducible nitric oxide synthase (iNOS or NOS2) appears to be largely competitive with O_2 (Brown and Borutaite, 2007).



ROS species can also interact with diverse macromolecules such as proteins, lipids and DNA, resulting in their damage (Figure 1.2) (Ott et al, 2007). Out of various free radical-induced biochemical processes, lipid peroxidation is the most extensively studied pathway. The methylene (-CH_2-) groups of polyunsaturated fatty acids (PUFA) are highly susceptible to oxidation. The oxidation of membrane phospholipids in the plasma membrane, as well as within internal organelle membranes such as mitochondria, leads to biophysical changes that disrupt membrane and organelle function (Valko et al, 2006). Lipid peroxidation in turn yields additional reactive species (e.g., 4-hydroxynonenal (4-HNE), malonaldehyde), which may contribute to toxicity and/or cellular signaling (Franco and Cidlowski, 2009).

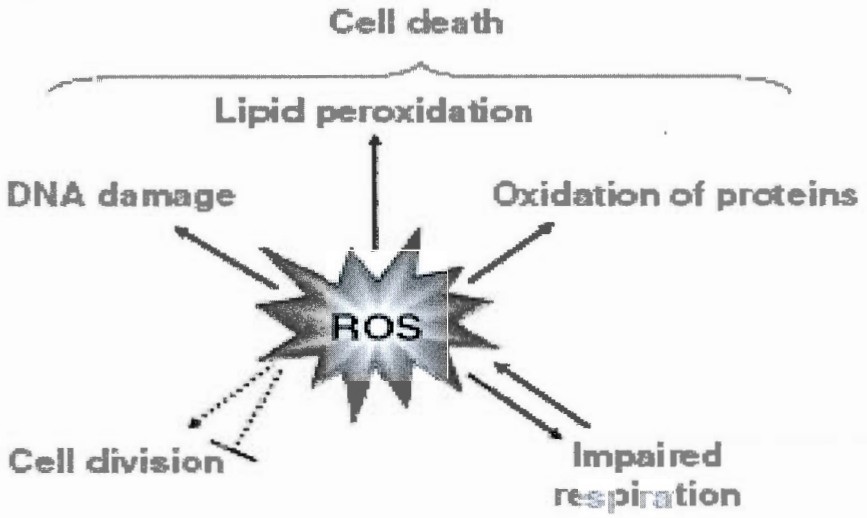


Figure 1.2 Damaging effects of ROS on key cell components. ROS cause damage to cell constituents, which include DNA, proteins and lipids. Depending on the levels of ROS, the cell either stimulates or inhibits cellular proliferation resulting in the initiation of cell death processes. Figure adapted from Ott et al. 2007.

1.2 ANTIOXIDANTS

1.2.1 Enzymatic antioxidants

Cells possess various defense mechanisms to prevent the damage caused by ROS. The major defense mechanisms include the enzymatic antioxidants superoxide dismutase (SOD), catalase and glutathione peroxidase (Figure 1.3).

(1) Cytoplasmic Copper/Zinc (Cu/Zn)-SOD, mitochondrial MnSOD, and extracellular SOD, catalyze conversion of $O_2^{\bullet -}$ to H_2O_2. H_2O_2 is lipid soluble and it can diffuse through membranes and generate the hydroxyl radical ($OH^{\bullet}$) at localized Fe^{2+}- or Cu^{+}- containing sites. It is also the precursor of hypochlorous acid (HOCl), a powerful oxidizing agent that is produced by phagocytic cells. Leukocytes and macrophages release myeloperoxidase that converts H_2O_2 to HOCl (Klebanoff, 1999).

(2) Catalase, present in peroxisomes, can neutralize hydrogen peroxide to water and oxygen ($2\ H_2O_2 \rightarrow H_2O + O_2$).

(3) Glutathione peroxidase (Gpx) can reduce cytoplasmic hydrogen peroxide to water with the help of reduced glutathione (GSH), which gets oxidized to glutathione di-sulfide (GSSG). The later can be reconverted to the reduced form by the enzyme glutathione reductase using a molecule of NADPH produced by the pentose phosphate pathway.

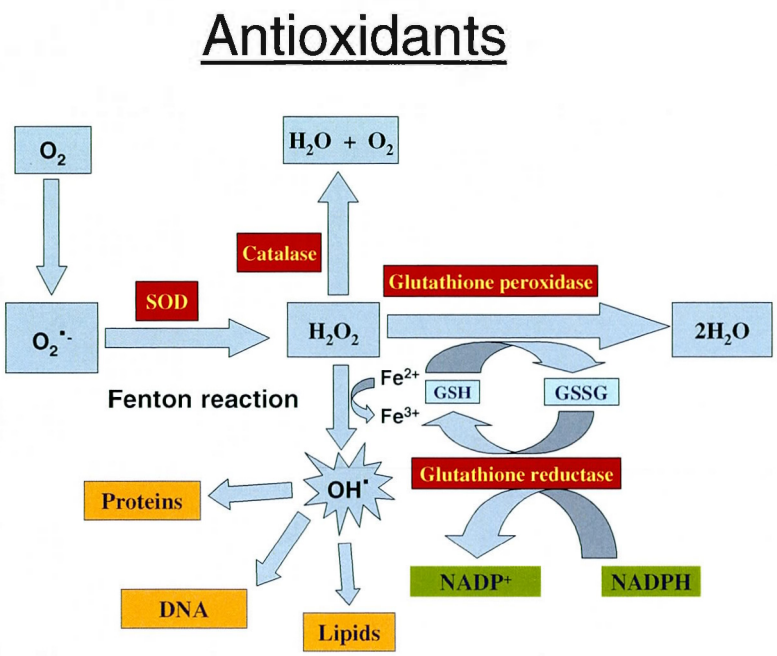


Figure 1.3 Role of enzymatic antioxidants in ROS detoxification. Generation of ROS can be triggered by various environmental factors (e.g. UV, IR radiations, and toxins) and by normal metabolic processes. ROS are kept in check by antioxidant defenses, which include detoxifying enzymes such as catalase, GSH peroxidase and SOD. If these antioxidant defence mechanisms are too weak, ROS-mediated damage to cellular macromolecules will eventually lead to cell death.

1.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants like glutathione (GSH), carotenoids, flavonoids, uric acid, bilirubin, α-tocopherol (vitamin E), ascorbic acid (vitamin C) and retinol (vitamin A) can prevent the oxidation of various cell components (e.g. unsaturated fatty acids) by free radicals such as superoxide and hydrogen peroxide by acting in one of the following ways: 1) they directly neutralise ROS, 2) they act as a chelators of transition metals such as iron and copper (e.g. uric acid) to decrease ROS production, or 3) they oppose the toxic actions of lipid peroxides and limit the amount of cellular lipid peroxide formation, and they also help by maintaining membrane integrity and homeostasis by repairing oxidative damaged lipid components (Valdo et al, 2007).

The balance between the generation of ROS and the cellular antioxidants determines the level of oxidative stress. When the intracellular oxidative activities overwhelm the reducing equivalents, it shifts cells into a condition of oxidative stress, leading to the activation of several stress signaling pathways (mitogen-activated protein kinases (MAPKs)/Extracellular signal-regulated kinases (ERK) pathway, stress-activated protein kinase (SAPK) pathways) and transcription factors (eg: NFκB, HSF-1, p53) that govern specific gene expression patterns (reviewed by Martindale and Holbrook, 2002; Finkel and Holbrook, 2000). Although the molecular mechanisms by which ROS initially activate these pathways are not yet clear, once activation occurs, these pathways lead to various cellular consequences, including proliferation, growth arrest, senescence and apoptosis.

1.3 OVERVIEW OF APOPTOSIS AND CELL DEATH

The term apoptosis was first introduced by Kerr and colleagues in 1972 (Kerr et al., 1972). It was named after an ancient Greek word, referring to the process of leaves falling from trees. Apoptosis is a genetically-controlled process of cell death, essential for the development and life of multicellular organisms (Prindull, 1995). It is an active, complex process with a characteristic set of morphological and biochemical features whereby cells undergo a cascade of self-destruction. Multicellular organisms often need to discard cells that are superfluous and potentially harmful, having accumulated mutations or become infected by pathogens. Thus, proper regulation of apoptosis is essential for maintaining normal cellular homeostasis. However, the deregulation of apoptosis is an underlying factor in many pathological conditions where excessive apoptosis of neurons due to toxic protein aggregates and oxidative stress has been implicated in Parkinson's and Alzheimer's diseases, and failure to execute apoptosis contributes to chronic inflammation, atherosclerosis and cancer (Thompson, 1995). Thus, it is highly important to understand the molecular mechanisms behind the regulation of apoptosis and determine which proteins are involved in this process.

The current classification of different types of cell death was reviewed recently by (Kroemer and Galluzzi 2009; Galluzzi et al, 2007). There are many observable morphological differences between the cell death phenotypes for apoptosis, necrosis, autophagy and mitotic catastrophe (Figure 1.4; Table 1.1). The mechanism (i.e. apoptosis, necrosis or autophagic cell death) by which a cell dies depends on different exogenous factors, the severity and duration of stress, the cell type, as well as the ability of the cell to handle the stress exposure. The important objective of this study is to understand the molecular mechanisms of apoptotic cell death induced by oxidative stress; hence it has been elaborated in detail.

Cell death mode	Characteristic morphological aspects
Apoptosis (Type 1)	● Rounding up of the cell ● Reduction of cellular and nuclear volume (pyknosis) ● Retraction of pseudopodes ● Nuclear fragmentation (karyorrhexis) ● Little modification of cytoplasmic organelles ● Plasma membrane blebbing
Autophagy (Type 2)	● Lack of chromatin condensation ● Massive vacuolization of the cytoplasm (double-membraned autophagic vacuoles)
Necrosis (oncosis) (Type 3)	● Cytoplasmic swelling ● Rupture of plasma membrane ● Swelling of cytoplasmic organelles ● Moderate chromatin condensation
Mitotic catastrophe	● Micronucleation ● Multinucleation

Table 1.1 Characteristic morphological features of distinct modalities of cell death. Table adapted from Galluzzi et al, 2007.

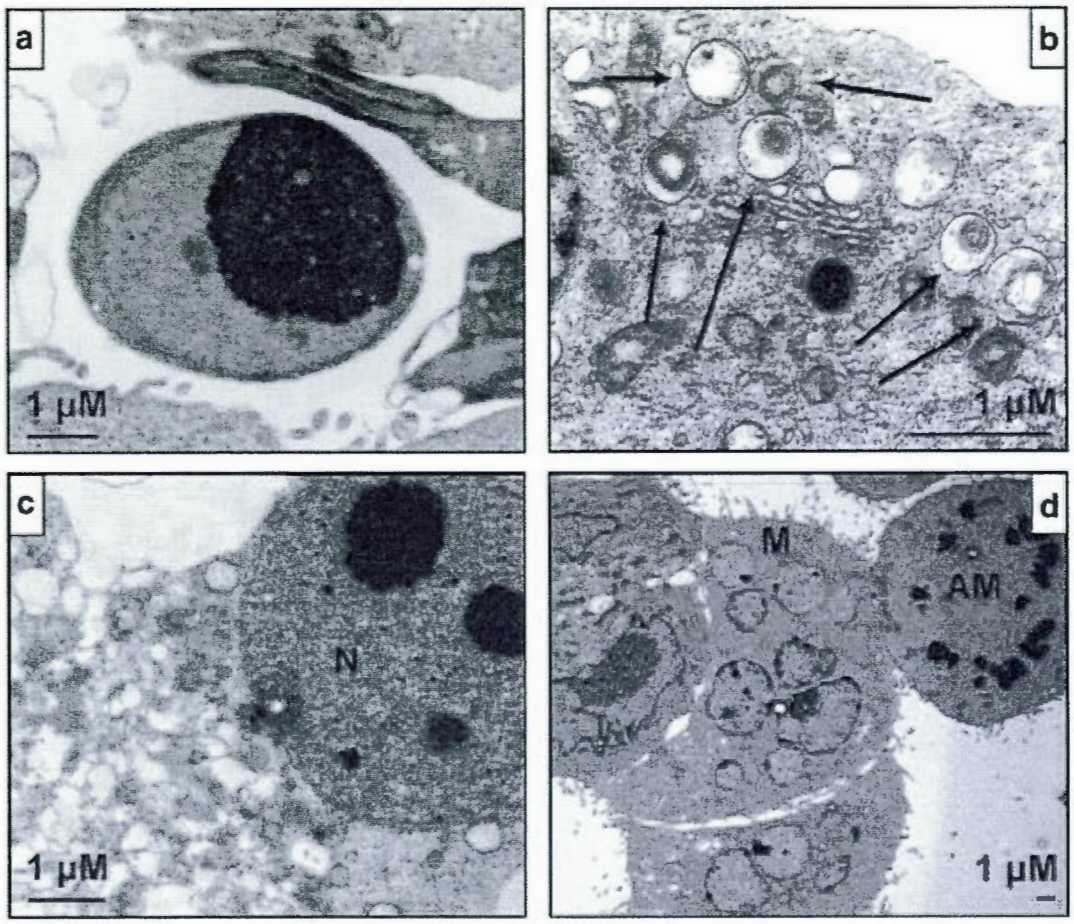


Figure 1.4 Different modes of cell death. Characteristic morphological features of distinct modes of cell death, seen by transmission electron microscopy. (a) H1975 (human non-small cell lung cancer) cell undergoing apoptotic cell death is represented by reduction of cellular and nuclear volume (pyknosis). (b) HeLa (human epithelial cancer) cell undergoing autophagic cell death is observed by the massive vacuolization of the cytoplasm and accumulation of double-membraned autophagic vacuoles (indicated by arrows). (c) HCT116 (human colon carcinoma) cell responding to a necrotic stimulus. The nucleus (N) remains intact and has clearly defined nucleoli, while the plasma membrane and cytoplasm are extensively damaged. (d) HCT116 cell exhibiting hallmarks of mitotic catastrophe, including aberrant multipolar mitotic figures (AM) and multinucleation (M). Figure adapted from Galluzzi et al. 2007.

Apoptosis can be triggered by a variety of exogenous factors (Wyllie, 2010). These include death signals, which can be physiological messenger molecules (e.g. cytokines, hormones), and stress induced by DNA damaging agents (e.g. UV and gamma irradiations), serum deprivation, ROS, reactive nitrogen species (RNS), hypoxia, heat shock, viral infection, as well as chemical (e.g. pesticides, environmental pollutants) and pharmacological agents (e.g. chemotherapeutic drugs). Furthermore, many apoptotic stimuli can themselves increase generation of ROS and RNS, leading to oxidative damage of cellular constituents and eventually cell death by apoptosis. A family of enzymes known as caspases plays a major role in the induction of apoptotic cell death.

1.3.1 Caspases

The family of cysteine proteases known as "Caspases" (cysteinyl aspartate-specific proteases), play an important role in apoptosis and inflammation (Pop and Salvesen, 2009). "C" in the term caspase stands for cysteine protease and "aspase" reflects their ability to cleave substrates at aspartic acid (Asp) residues (Lavrik et al, 2005) in the context of tetrapeptide motifs. They are synthesized as catalytically inactive zymogens. These proteases typically lie dormant in the healthy cell and, in response to cell-death stimuli, are converted, either by proteolytic cleavage or by recruitment into large complexes, into active enzymes. The constitutively expressed procaspases consists of three domains: an NH_2-terminal pro-domain, a large (~20 kDa) and a small (~10 kDa) domain. The large 20 kDa domain contains the active site cysteine within a conserved QACXG motif. The activation of caspases requires the cleavage of specific aspartic acid residues, which separates the N-terminal prodomain, the p10 domain and the p20 domain. When processed, the two subunits (p10 and p20) will associate and form a heterodimer. The active form of caspase is a heterotetrameric enzyme consisting of two large and two small subunits (Figure 1.5) with two active catalytic sites per molecule (Kohler et al, 2002).

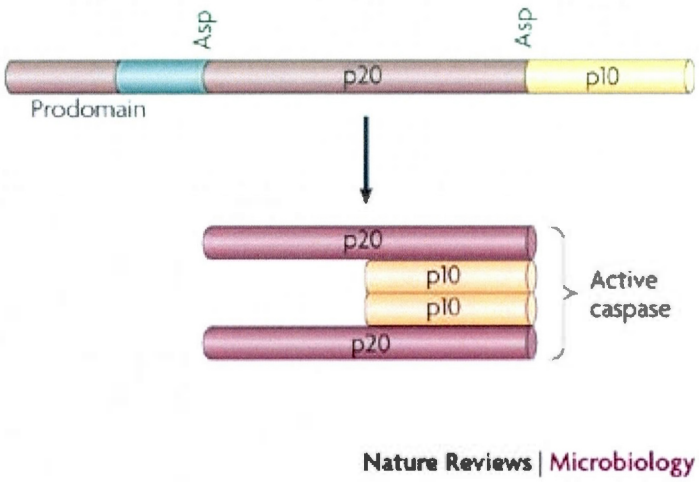


Figure 1.5 Caspase activation by proteolysis. Caspases are synthesized as inactive zymogens/procaspases that are activated by proteolysis. Caspase activation requires proteolytic processing (two cleavages) at specific aspartic acid (Asp) residues. The first divides the proenzyme into large and small caspase subunits, and the second removes the N-terminal prodomain. The resulting functional caspase is a tetramer of two large (p20) and two small (p10) subunits. Figure adapted from Clarke et al, 2009.

Fourteen caspases have been identified so far, based on their homology in amino acid sequences and substrate specificity; these caspases can be classified into apoptotic (caspases-2, -3, -6, -7, -8, -9, -10) and pro-inflammatory (caspases-1, -4, -5, -11, -12, -13 and -14) groups. Apoptotic caspases can be subdivided further into initiator (apical, apoptosis activator) (caspases-8, -9 and -10) and effector (executioner, downstream) groups (caspases-3, -6 and -7) (Table 1.2).

Subfamily	Role	Members
I	Apoptosis activator	Caspase-2
		Caspase-8
		Caspase-9
		Caspase-10
II	Apoptosis executioner	Caspase-3
		Caspase-6
		Caspase-7
III	Inflammatory mediator	Caspase-1
		Caspase-4
		Caspase-5
		Caspase-11
		Caspase-12
		Caspase-13
		Caspase-14

Table 1.2 Subfamily members of the caspase family. Table adapted from Fan et al, 2005.

The most significant structural difference regarding caspases involves the varying length of the N-terminal prodomain. The initiator caspases such as caspase-8 and -9 have longer prodomains that allow them to interact with death effector domains (DEDs) or caspase recruitment domains (CARDs) in adaptor proteins such as Fas-associated death domain (FADD) protein and apoptotic protease-activating factor -1 (Apaf-1). Effector caspases (such as caspase-3) contain short pro-domains and they function primarily to cause the morphological features of apoptosis (Kohler et al, 2002). In response to an apoptotic stimulus, initiator caspases are the first to be activated. These active initiator caspases in turn process and activate effector procaspases. Once activated, they subsequently cleave many intracellular substrates, including structural proteins such as

lamin A, actin, gelsolin and fodrin, the DNA repair enzyme poly (ADP-ribose) polymerase (PARP), cell cycle regulators such as p21, Rb and mdm2, and the inhibitor of caspase-activated DNase (ICAD) (Figure 1.6) (Fan et al, 2005). Caspase-activated deoxyribonuclease (CAD) is one of the key substrates for activated caspase-3. It normally exits as an inactive enzyme bound to its inhibitor ICAD (inhibitor of caspase-activated DNase). Active caspase-3 cleaves ICAD, thereby releasing CAD, which cleaves cellular DNA, resulting in DNA fragmentation. Another important target for activated caspase-3 is the nuclear protein PARP (molecular mass 116 kDa). PARP plays a key role in the maintenance of chromatin structure as well as DNA repair. Active caspase-3 cleaves PARP to an 85 kDa fragment, which hinders its DNA-repairing activity (Wang et al, 2005). Cleavage of ICAD, nuclear and cytoskeletal proteins leads to characteristic morphological hallmarks of apoptosis such as chromatin condensation, nucleosomal DNA fragmentation, nuclear membrane breakdown, externalization of phosphatidylserine, and the formation of apoptotic bodies (Hengartner 2000). Apoptotic bodies are removed by phagocytic or neighbouring cells without releasing intracellular contents into surrounding tissue, thus avoiding induction of inflammatory responses. Addition to their function in cell death recent publications also provide increasing evidence that apoptotic caspases also participate in several non-apoptotic cellular processes such as cell proliferation, differentiation, shaping and migration (Kuranaga, 2011; Kuranaga and Miura, 2007).

The other important family of cysteine proteases that is calpains also plays an important role in initiation, regulation and execution of cell death (Harwood et al, 2005), but their role is not as well characterized as that for the caspases. Calpains are also known to cleave many important regulators of apoptosis and to crosstalk with caspase cascades. All the above mentioned intracellular proteins, such as lamins, actins, fodrin, and PARP, also act as substrates for active calpains and thus play an important role in the execution of apoptosis (reviewed by Lopatniuk and Witkowski, 2011).

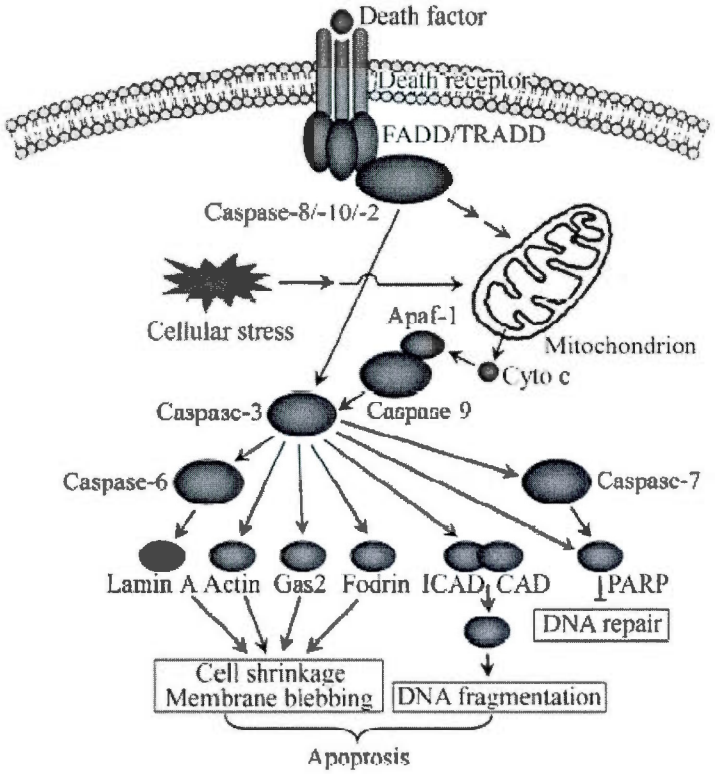


Figure 1.6 Downstream substrates that are targeted by active effector caspases, resulting in the execution of characteristic morphological features of apoptosis. Figure adapted from Fan et al, 2005.

1.3.2 Signaling pathways of Apoptosis

Cells undergo apoptosis mediated by three major signaling pathways, involving death receptors (extrinsic pathway), mitochondria (intrinsic pathway), and more recently, the endoplasmic reticulum (ER) (intrinsic) (Strasser et al, 2009; Fulda et al, 2010; Duprez et al, 2009).

1.3.2.1 Mitochondrial (intrinsic) pathway

Mitochondria are important intracellular organelles for the production of ATP by oxidative phosphorylation, which supplies energy for various cellular functions. In addition to supplying energy, mitochondria play a critical role in the initiation and regulation of apoptosis. A variety of stress-inducing agents such as aldehydes (e.g. acrolein), chemotherapeutic agents, UV and gamma irradiations, heat shock and growth factor withdrawal can trigger apoptosis through the mitochondrial pathway (Jin et al., 2005; Tanel and Averill-Bates, 2005; Bettaieb and Averill-Bates, 2005).

A critical cellular target of ROS is mitochondrial DNA (mtDNA), which could lead to lethal cell injury by disrupting electron transport, mitochondrial membrane potential and ATP generation (Ott et al, 2007). Therefore, ROS can cause mitochondrial dysfunction, leading to activation of the mitochondrial pathway of apoptosis (Orrenius et al, 2007). Induction of the mitochondrial permeability transition (MPT) and mitochondrial outer membrane permeabilization are important features of apoptosis. A consequence of the MPT is swelling of the matrix and rupture of the outer mitochondrial membrane. These changes lead to the release of cytochrome c (Cyt c), and other pro-apoptotic mitochondrial proteins such as DIABLO/SMAC, Omi/Htr2, apoptosis inducing factor (AIF) and endonuclease G (Endo G) into the cytoplasm, resulting in cell death by both caspase-dependent and caspase-independent mechanisms (Figure 1.7).

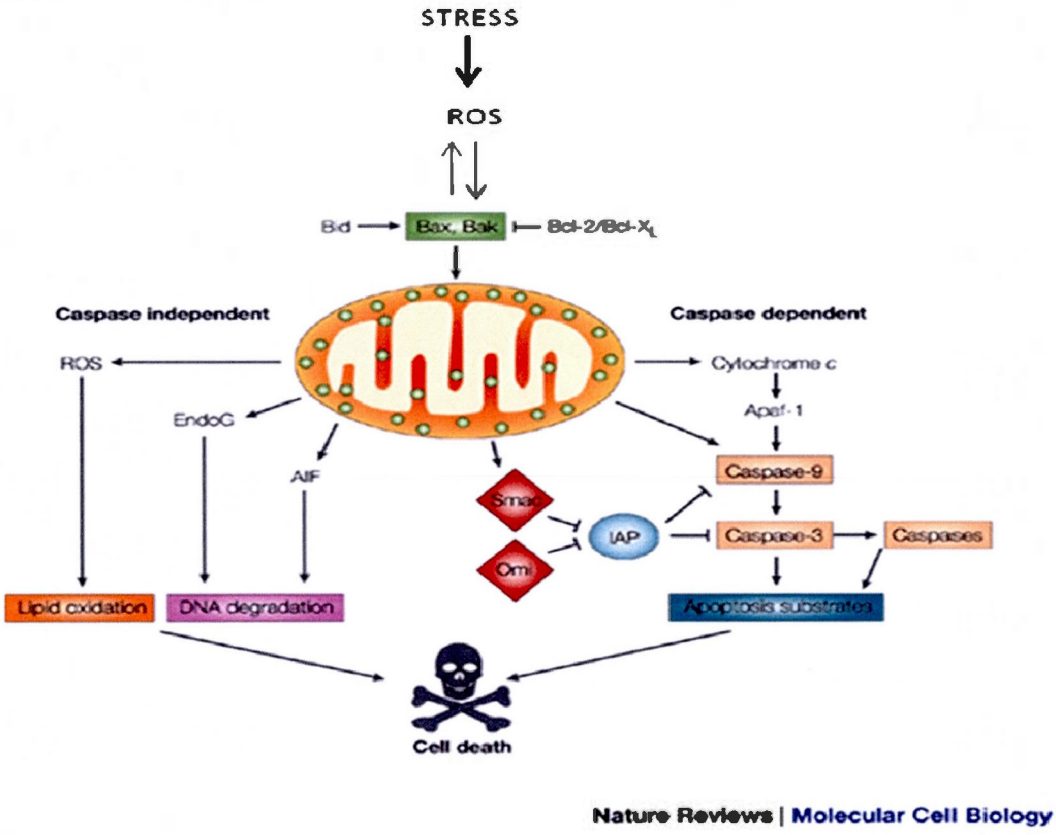


Figure 1.7 Caspase-dependent and -independent pathways of apoptotic cell death. Various apoptotic stimuli (e.g., ROS) cause permeabilization of the mitochondrial outer membrane and the release of proapoptotic proteins. In the cytosol, cytochrome c, Apaf-1 and dATP form the apoptosome complex, to which initiator procaspase-9 is recruited and activated. Caspase-9-catalyzed activation of effector caspase-3 executes the final steps of apoptosis. Caspase activation is further enhanced by neutralization of caspase inhibitors by apoptogenic proteins such as Smac/Diablo and Omi/HtrA2 that are released from mitochondria. In addition, mitochondrial proteins such as AIF and Endo G promote caspase-independent apoptosis through nuclear translocation and by mediating genomic DNA fragmentation. Figure adapted from Jin et al., 2002 (modified).

The haemoprotein cytochrome c plays an important role in caspase-dependent apoptosis. Under normal conditions, cytochrome c is a component of the respiratory chain and is attached to the outer surface of the inner mitochondrial membrane (IMM), mainly in association with the anionic phospholipid cardiolipin (CL) (Ott et al., 2007). The oxidation of cardiolipin by ROS decreases cytochrome c binding and increases the level of soluble cytochrome c in the intermembrane space (Orrenius et al., 2007) (Figure 1.8). Once released, cytochrome c binds to Apaf-1 and procaspase-9, generating an intracellular disc-like complex known as the "apoptosome". This leads to the auto-activation of caspase-9, which can subsequently activate the executioner caspases such as caspase-3, -6 and -7, resulting in the ultimate demise of the cell.

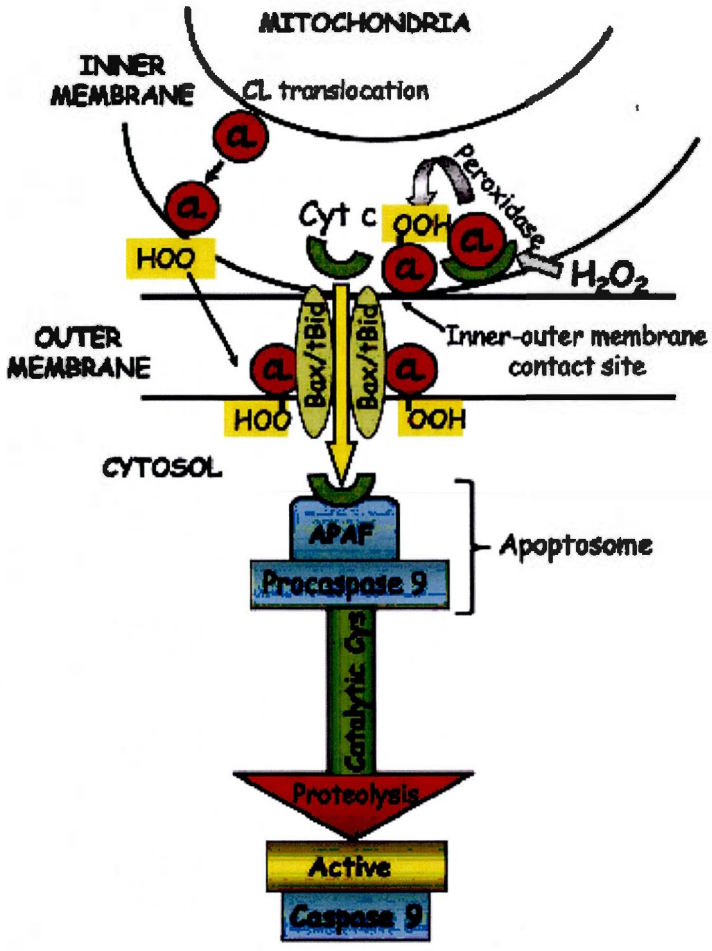


Figure 1.8 Effects of ROS on cardiolipin oxidation and cytochrome c release into the cytosol. Normally cytochrome c (Cyt c) is bound to the mitochondrial inner membrane by an interaction with the anionic phospholipid cardiolipin (CL). Oxidation of cardiolipin by mitochondrial ROS (H_2O_2) decreases its binding affinity for cytochrome c, which results in cytochrome c detachment. Oxidized cardiolipin, together with pro-apoptotic proteins (e.g. Bid and Bax), forms a megapore channel within the mitochondrial outermembrane, which enables cytochrome c release from mitochondria into the cytosol. In the cytosol, cytochrome c interacts with Apaf-1, procaspase-9 and dATP, forming the apoptosome complex, resulting in caspase-9 activation. Figure adapted from Circu and Aw 2010.

The pro-apoptotic proteins second mitochondrial activator of caspases (SMAC)/ direct inhibitor of apoptosis-binding protein with low pI (DIABLO), and high-temperature requirement A2 serine protease (HtrA2/Omi), which are released from mitochondria during apoptosis, facilitate caspase activation by binding to and neutralizing the anti-apoptotic activity of inhibitors of apoptosis proteins (IAPs), which are potent caspase inhibitors (Figure 1.7).

Once released from mitochondria, AIF and Endo G are involved in caspase-independent apoptosis (Figure 1.7). These proteins can bind directly to DNA, which can stimulate their DNAse activity, leading to chromatin condensation and large-scale DNA fragmentation. AIF appears to play a role in mediating cell death in several human disease states, including ischemia-reperfusion injury, neurodegenerative disorders and certain types of cancers (Norberg et al, 2010).

1.3.2.2 Death receptor (extrinsic) pathway

The extrinsic pathway involves the binding of external ligands to death receptors located at the plasma membrane. These death receptors belong to the tumor necrosis factor receptor (TNF-R) super family and generally have several cellular functions including inflammation and apoptosis (Baud and Karin, 2001). The death receptors that are involved in apoptosis possess an intracellular death domain (DD). They include TNF receptor 1 (TNFR1 (also known as death receptor 1 (DR1)), Fas receptor (DR2, CD95 or APO-1), DR3 (APO-3 or TRAMP), TNF-Related Apoptosis-Inducing Ligand (TRAIL) receptor 1 (TRAIL-R1; also called DR4), TRAIL receptor 2 (TRAIL-R2; also called DR5 or KILLER) and DR6 (Russo et al, 2010).

The Fas ligand (FasL)/Fas system is the most widely studied system in death-receptor-mediated apoptosis. Fas-mediated apoptosis is activated by the binding of FasL to its

specific receptor Fas. This interaction results in clustering and trimerization of the receptor, which is required for formation of the DISC at the plasma membrane. The DD of the receptor interacts with the DD of FADD, while the DED of FADD interacts with the DED domains of procaspase-8 or procaspase-10. The association of procaspase-8 or procaspase-10 at the DISC leads to their auto-activation (Elrod et al, 2008). A significant level of caspase-8 activation directly activates effector caspases such as caspase-3, -6 and -7, whereas low caspase-8 activation mediates caspase-3 activation through an amplification loop involving mitochondria (Figure 1.9). Thus, the death receptor and mitochondrial pathways can be linked through caspase-8-mediated cleavage of pro-apoptotic Bcl-2 family protein Bid. The cleaved fragment, t-Bid, then translocates to mitochondria and induces outer mitochondrial membrane permeabilization through its interaction with Bcl-2 family proteins such as Bax and Bak. This interaction results in the release of cytochrome c into the cytosol, which is a key step required for the induction of mitochondrial-mediated apoptosis (Youle and Strasser, 2008) (Figure 1.9).

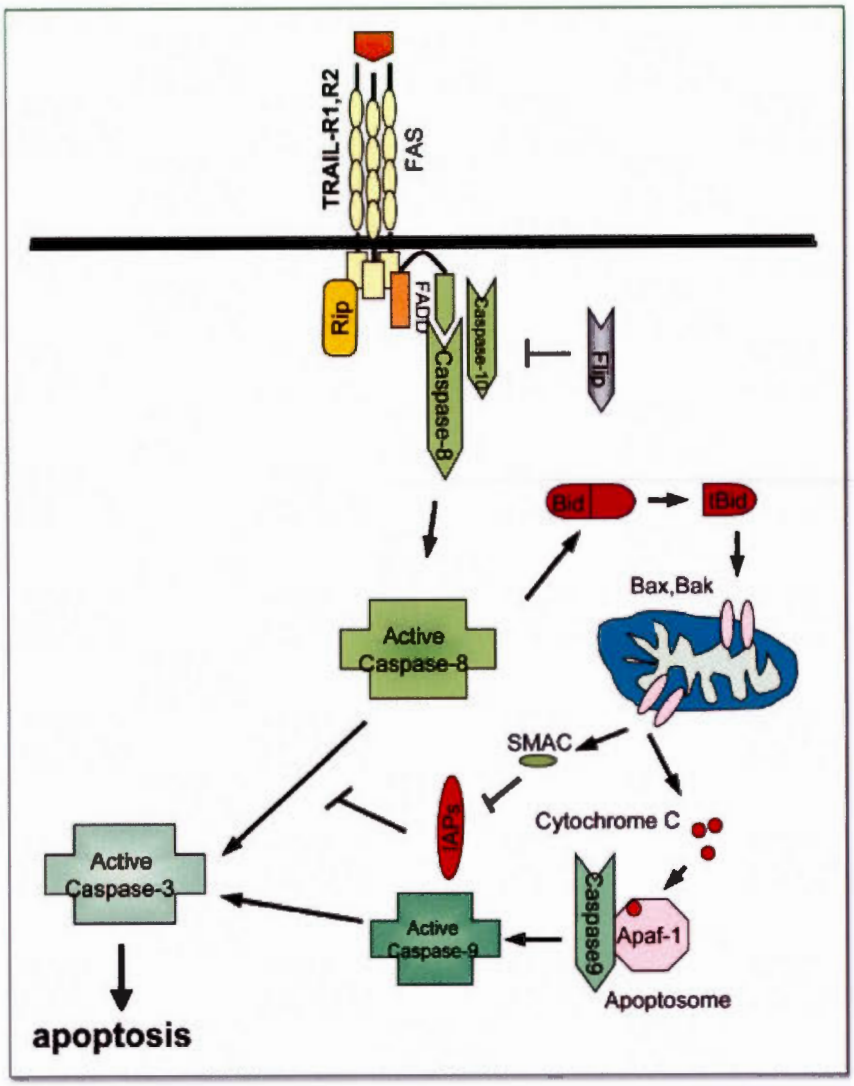


Figure 1.9 Fas- mediated death receptor pathway of apoptosis. Figure adapted from Zhaoyu et al 2005 (see text for details).

Fas can also mediate apoptosis via death domain associated protein (Daxx (also known as Death-associated protein 6)), with subsequent activation of apoptosis signal-regulating kinase 1 (ASK-1) and c-Jun N-terminal kinase (JNK) (Dorion et al, 2002; Salomoni and Khelifi, 2006; Fujisawa et al, 2007). During apoptosis, Daxx translocates from the nucleus to the plasma membrane and specifically binds to the death domain of Fas. Although Daxx does not contain a death domain, it plays a central role in Fas-mediated apoptosis through activation of ASK-1. ASK-1 is an upstream regulator of the JNK and p38 mitogen-activated protein kinases (MAPKs) during exposure to stressful stimuli such as hyperosmotic shock, oxidative stress, cold shock, UV irradiation, TNF-α and interleukin-1 (Fusijawa et al, 2007). JNK activation was shown to promote apoptosis by reducing the anti-apoptotic activity of Bcl-2 by causing its phosphorylation (Liu and Lin, 2005).

The induction of apoptosis by TRAIL is similar to that of Fas (Russo et al, 2010; Jin and El-Deiry, 2005). The binding of TRAIL-R1 and –R2 to their respective receptors, DR4 and DR5, triggers formation of the DISC by recruiting FADD and procaspase-8, or -10, followed by activation of caspase-3. TRAIL-induced apoptosis also involves amplification through the mitochondrial pathway, similar to Fas-induced apoptosis.

Unlike Fas and TRAIL signaling, the cytokine TNF-α does not induce cell death spontaneously. TNF-R1-mediated intracellular signaling is more complex than that of Fas or TRAIL-R1 and –R2 since it can activate both apoptotic and survival signals (Russo et al, 2010). Upon activation by TNF-α, trimerization of TNF-R1 is followed by recruitment of the adaptor protein TNFR-associated protein with death domain (TRADD). This results in the formation of two complexes that activate distinct downstream survival or apoptotic signaling pathways. TRADD recruits signaling proteins like FADD, TNF-associated factor-2 (TRAF-2), and receptor-interacting protein (RIP). In complex I, the interaction of TNF-R1 with TRADD, RIP and TRAF-2 leads to the activation of

transcription factors such as nuclear factor kappa B (NF-κB) and JNK, which can promote cell survival and the activation of immune and inflammatory responses (Deng et al, 2003; Beyaert R et al, 2002). Activation of NF-κB by TNF-α up-regulates several anti-apoptotic factors, including c-FLIP, Bcl-X_L, MnSOD, X-linked inhibitor of apoptosis protein (XIAP), and c-IAP 1 and 2.

Complex II is comprised of TNF-R1, TRADD, FADD and procaspase-8 and promotes apoptosis through the direct activation of effector caspases such as caspase-3 (Russo et al, 2010) (Figure 1.10). The cellular balance between $FLIP_L$ and RIP1 appears to be an important factor in determining whether TNF-R1 activation results in apoptosis or survival signalling. High concentrations of $FLIP_L$ inhibit procaspase-8 binding at complex II and prevent DISC formation, whereas caspase-8 mediated cleavage of RIP1 dissembles complex I and promotes the formation of complex II that mediates apoptosis (Circu and Aw, 2010).

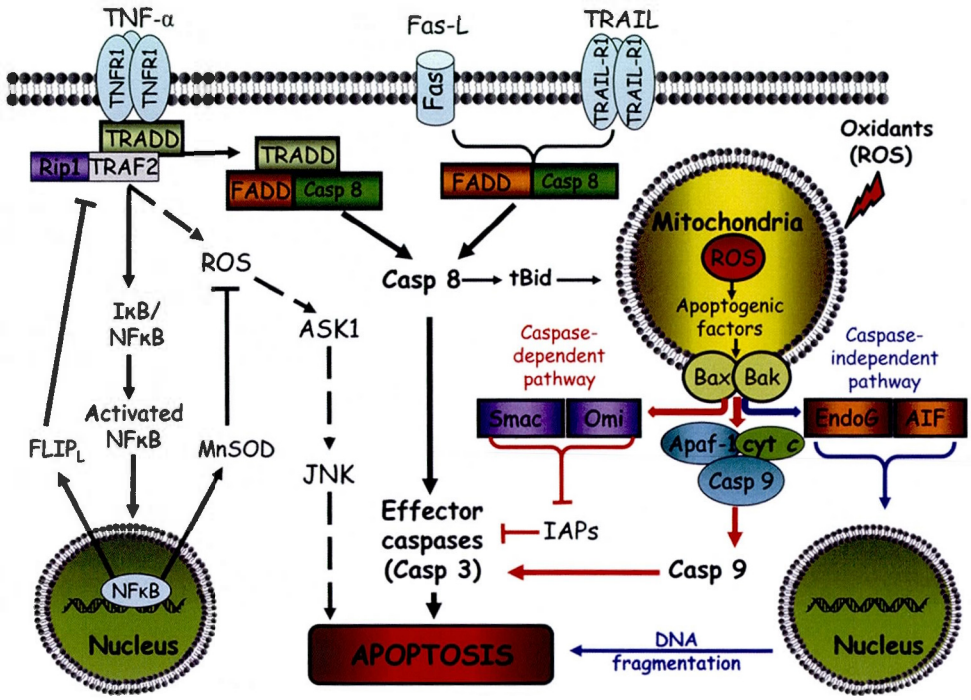


Figure 1.10 Apoptosis-mediated by the death receptors. Stimulation of death receptors such as Fas/FasL, TNF-R1/TNFα, and TRAIL-R1/TRAIL results in receptor aggregation and recruitment of adaptor molecules FADD/TRADD and caspase-8. Once recruited, caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases. The mitochondrial pathway is initiated by stress signals through the release of apoptogenic factors such as cytochrome c, AIF, or Smac/DIABLO. Cytochrome c release into the cytosol triggers caspase-3 activation through formation of the apoptosome complex. Smac/DIABLO promotes caspase activation by neutralizing the inhibitory effects of IAPs, whereas AIF causes DNA condensation. The receptor and the mitochondrial pathways can be interconnected, for example, by Bid, which causes cytochrome c release upon cleavage by caspase-8. Activation of caspases is negatively regulated at the receptor level by FLIP, which blocks caspase-8 activation, at the mitochondria by Bcl-2 family proteins, and by IAPs. Activation of the NF-κB survival pathway enhances transcription of antiapoptotic proteins such as $FLIP_L$ or MnSOD and apoptosis blockade. At high ROS levels, failure to activate NF-κB promotes ASK1/JNK activation that triggers apoptosis. Figure adapted from Circu and Aw, 2010.

Despite the early discovery of caspase-2, its physiological role remains unclear (Krumschnabel et al, 2009). Caspase-2 appears to have multiple roles in processes such as apoptosis, DNA repair and tumor suppression. Caspase-2 activation has been demonstrated in apoptosis induced by different systems such as anti-Fas, cytokine deprivation, β-amyloid, etoposide and other stress stimuli (Zhivotovsky and Orrenius, 2005). Several studies have reported p53-mediated activation of caspase-2 by the PIDDosome, in response to DNA damage (Maiuri et al, 2007; Tinel et al, 2007). The PIDDosome is a ternary complex that is composed of p53-induced protein with death domain (PIDD), RIP-associated Ich-1/Ced-3-homologue protein with a death domain (RAIDD) and procaspase-2. However, it was also shown that recruitment of caspase-8 to the DISC can lead to caspase-2 activation in the absence of PIDDosome formation (Lavrik et al, 2006; Manzl et al, 2009; Olsson et al, 2009). Thus, there exist different routes by which caspase-2 can be activated. Once activated, caspase-2 can trigger Bid-mediated activation of the mitochondrial pathway of apoptosis (Lavrik et al, 2006).

1.3.2.3 Endoplasmic reticulum (ER) stress (intrinsic) pathway

The ER is an organelle that is involved in lipid synthesis, protein folding and maturation (Breckenridge et al, 2003). Membrane and secretory proteins undergo folding and modification into their mature conformation in the ER and then are transported to lysosomes, Golgi and the cell membrane. Protein chaperones such as Bip/glucose-related protein 78 (GRP78), GRP94, GRP170, calnexin, calreticulin and the oxidoreductase protein disulphide isomerase (PDI) are present in the ER to assure correct folding of newly synthesized proteins (Figure 1.11).

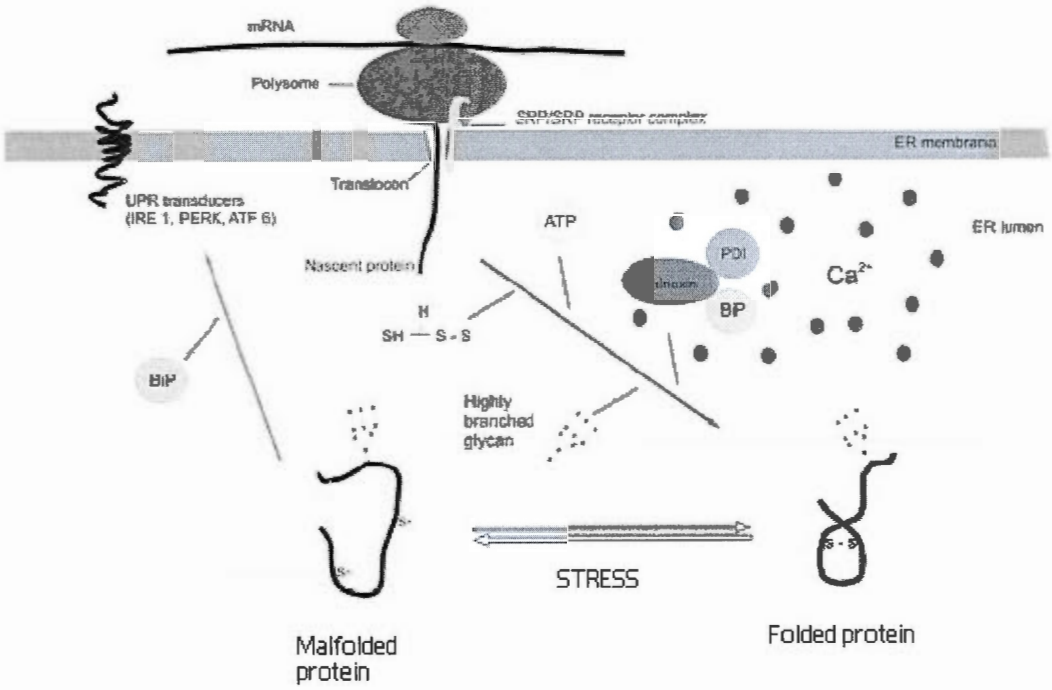


Figure 1.11 Functions of ER. Nascent proteins are translocated into the ER lumen, through protein channels in the ER membrane called translocons. Proper folding of nascent proteins is facilitated by ER chaperones including Bip, calnexin and protein disulphide isomerise (PDI). These chaperones facilitate proper folding of the nascent protein by preventing aggregation, and by maintaining polypeptides in appropriate conformations for folding and subunit assembly. During stress conditions, changes in the ER environment shift the balance from normal to improper folding, leading to accumulation of unfolded proteins in the ER lumen. This results in activation of the ER stress response pathway. Figure adapted from Faitova et al, 2006 (modified).

1.3.2.3.1 ER Stress

A variety of conditions including glucose deprivation, inhibition of protein glycolysation, disturbance of calcium homeostasis, hypoxia and excess ROS can perturb ER function leading to accumulation of unfolded proteins in the ER (Schroder and Kaufman, 2005; Fulda et al, 2010). This phenomenon is known as ER stress. ER stress activates several signaling pathways, including the unfolded protein response (UPR) and ER-associated protein degradation (ERAD). The UPR is generally a survival response that aims to recover normal cellular function in the face of adverse conditions. However, if protein damage is too severe, ER stress plays an important role in elimination of the damaged cell by apoptosis and/or autophagy (Schleicher et al, 2010; Verfaillie et al, 2010).

The ER stress response involves 3 distinct mechanisms: (i) translational attenuation to decrease the synthesis of new proteins; (ii) transcriptional activation of genes for ER chaperones (eg: Bip, GRP94), ERAD molecules and antioxidants; and (iii) ERAD, which involves translocation of misfolded or aggregated ER proteins to the cytoplasm, where they are tagged by ubiquitin for proteosomal degradation (Shroder and Kaufman, 2005; Verfaillie et al, 2010).

The activation of the UPR is mediated by three distinct ER stress sensors: Inositol-requiring protein-1(IRE1), activating transcription factor-6 (ATF6) and protein kinase RNA (PKR)-like ER kinase (PERK) (Figure 1.12). In non-stressed cells, these sensors are retained in the ER lumen by interactions with Bip/GRP78 (Shroder and Kaufman, 2005). During ER stress, Bip releases these 3 sensors, leading to their activation, and then binds to unfolded proteins. PERK and IRE1 undergo homo-oligomerization and autophosphorylation during activation (Figure 1.12).

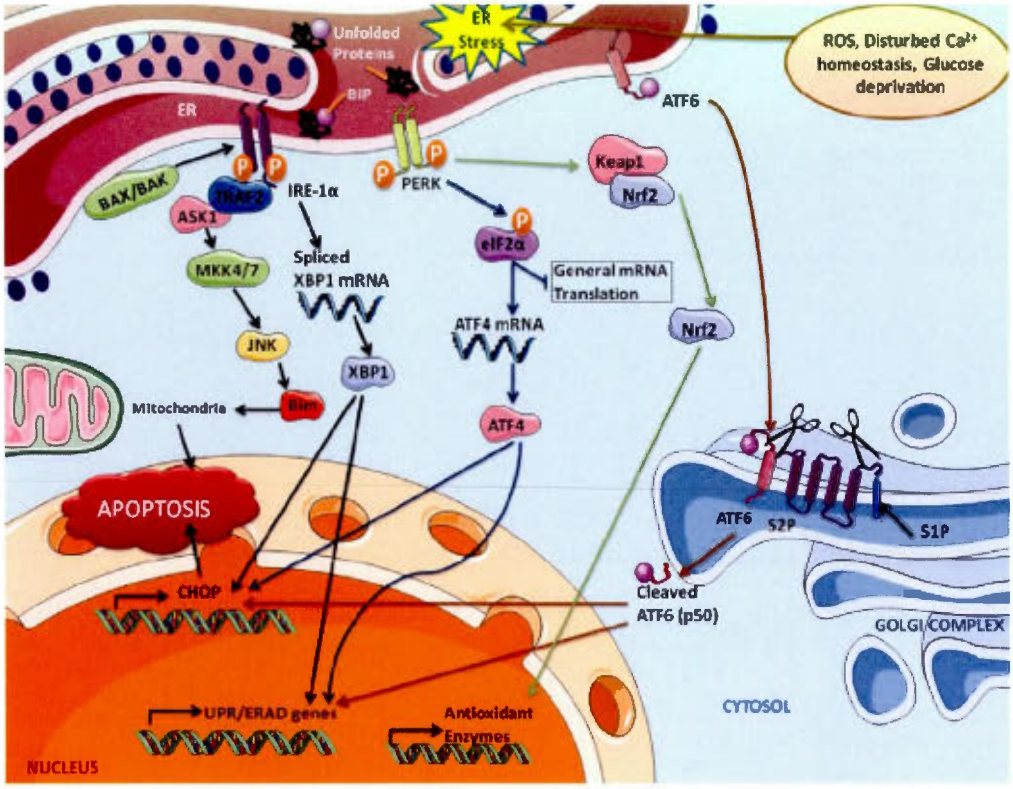


Figure 1.12 Signaling the ER stress response (UPR) pathway. Accumulation of unfolded proteins in the ER leads to activation of three ER stress sensors PERK, IRE1 and ATF6. Activation of these sensors is triggered by dissociation of Bip from their ER luminal domains. Once activated, PERK phosphorylates the translation initiation factor eIF2α to prevent further accumulation of unfolded proteins. Phosphorylation of NF-E2-related factor 2 (Nrf2) by PERK, promotes its dissociation from Kelch-like Ech-asspciated protein 1 (Keap1), leading to nuclear accumulation of Nrf2. Nrf2 binds to the antioxidant response element (ARE) to activate transcription of genes encoding detoxifying enzymes (antioxidants). Activated IRE1 splices the mRNA for XBP-1, which allows the translation of mature XBP-1 protein, a transcription factor, responsible for the upregulation of genes involved in ERAD, ER quality control and redox homeostasis. ATF6 is activated by a two-step cleavage by site-1 and site-2 proteases (S1P and S2P). The cleaved fragment (p50) forms an active transcription factor that mainly mediates expression of several components for ERAD and ER homeostasis. When functions of the ER are severely impaired, apoptosis is induced by the transcriptional induction of CCAAT/-enhancer-binding protein homologous protein (CHOP), which can be up-regulated by XBP1, ATF6 and PERK, and can mediate transcription of the pro-apoptotic BH3-only protein Bim. Figure adapted from Verfaillie et al, 2010.

Activated PERK then phosphorylates the eukaryotic initiation factor 2 (eIF2α), which inhibits general protein translation, thus preventing the accumulation of newly synthesized proteins in the ER under conditions where protein folding is compromised. The PERK-eIF2α pathway causes translation of ATF4, which activates transcription of chaperones such as Bip and GRP94. IRE1 catalyzes the alternative splicing of the X-Box binding protein (XBP-1) mRNA, leading to expression of the active XBP1 transcription factor that stimulates transcription of ER chaperone genes (Calfon et al, 2002). The up-regulation of ER chaperone genes is also mediated by the ATF6 pathway. ER stress causes translocation of ATF6 from the ER to Golgi, where it is processed by 2-step cleavage, by Site-1 protease (S1P) and Site-2 protease (S2P). The cleaved active N-terminal fragment of ATF6 (p50) is then transported to the nucleus, where it functions as a transcriptional activator for ER stress-related genes (Breckenridge et al, 2003). If UPR activation is not able to rescue cells from ER stress and correct the defects in protein folding, then cells generally undergo ER stress-induced cell death (Breckenridge et al, 2003; Fulda et al, 2010; Malhotra and Kaufman, 2007a; Rasheva and Domingos, 2009).

1.3.2.3.2 ER Stress-mediated apoptosis

Cells undergo ER-mediated apoptosis by several different pathways, which can be mitochondria-independent or -dependent:

(1) The caspase-12/caspase-4 pathway; (2) IRE1-mediated activation of apoptosis signal-regulating kinase 1 (ASK1)/JNK; (3) Bax/Bak-regulated calcium release from the ER (Figure 1.13); (4) PERK/ATF6/IRE1-dependent transcriptional induction of the pro-apoptotic transcription factor CHOP; and (5) Cleavage of transmembrane ER protein Bcl-2-associated protein-31 (BAP31) by active caspase-8.

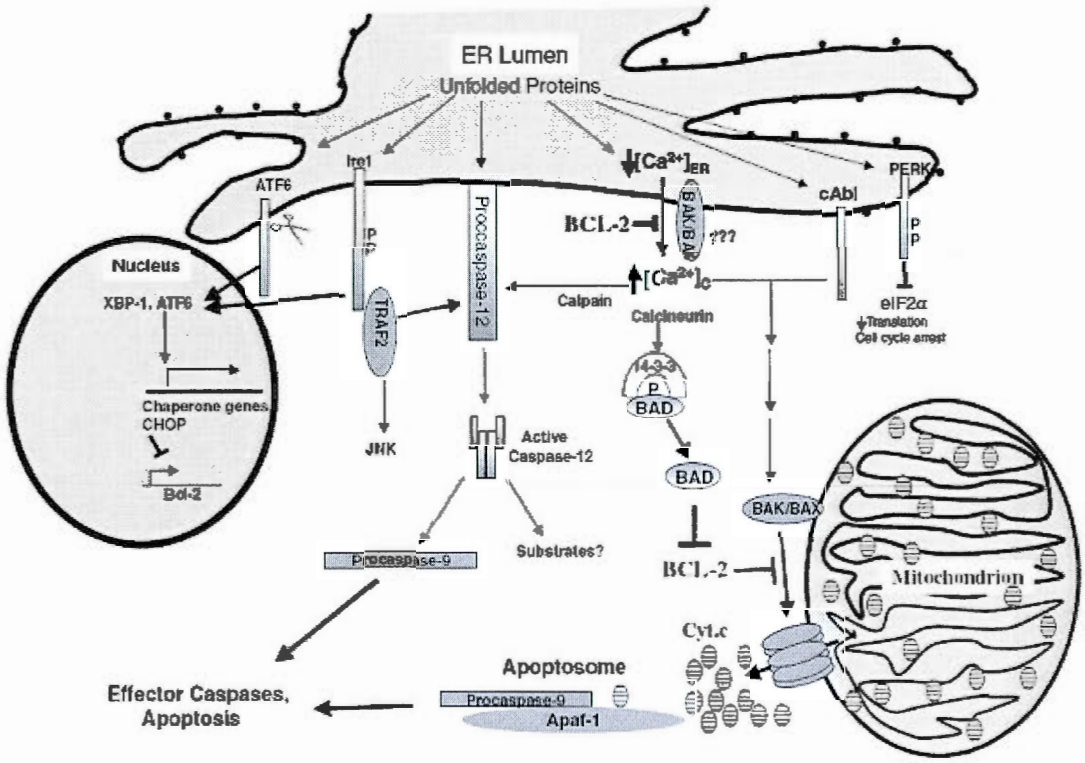


Figure 1.13 Mechanism of the ER mediated apoptosis. Figure adapted from Breckenridge et al, 2003.

(1) Caspase-12 (mice) and -4 (human) have been proposed as caspases that initiate ER stress-induced apoptosis (Fulda et al, 2010; Malhotra and Kaufman, 2007a). Procaspase-12 is localized at the cytosolic side of the ER membrane and can be activated through several mechanisms, including cleavage by the calcium-dependent cysteine protease m-calpain (Boyce and Yuan, 2006) (Figure 1.13). Therefore, calcium released from the ER can trigger apoptosis through calcium-mediated calpain activation and crosstalk between the calpain and caspase families. Caspase-12 can also be cleaved by caspase-7, which is recruited to the ER during ER stress (Boyce and Yuan, 2006). Caspase-7 forms a complex with caspase-12 and Bip, resulting in caspase-7-mediated cleavage of caspase-12.

Furthermore, caspase-12 appears to form a complex with IRE1 and TRAF2, which provides a scaffold for caspase-12 processing during ER stress (Yoneda et al, 2001) (Figure 1.13). Once activated, caspase-12 can activate caspase-9, which in turn activates caspase-3, in a manner that is independent of the cytochrome c/Apaf-1 pathway (Szegezdi et al, 2003). However, the role of caspase-12 in ER stress-induced apoptosis is controversial, since most of the human population does not possess the complete functional gene product of caspase 12 (Boyce and Yuan, 2006). Instead, caspase-4 has been suggested to be the human counterpart of murine caspase-12.

(2) ER stress-induced apoptosis can be mediated by interaction between IRE1 and the adaptor protein TRAF2 (Malhotra and Kaufmann, 2007a; Boyce and Yuan, 2006). IRE1 and TRAF2 then interact with ASK1, which phosphorylates and activates JNK (Figure 1.12). Once activated, JNK can phosphorylate and inactivate the anti-apoptotic protein Bcl-2 at the ER membrane leading to its proteasomal degradation. Consequently, phosphorylated Bcl-2 is unable to inhibit pro-apoptotic BH3-only proteins (Bax and Bak) and loses its ability to regulate ER Ca^{2+} fluxes (Bassik et al, 2004). Phosphorylated Bcl-2 enhances calcium efflux from the ER, which leads to calcium uptake by mitochondria. In addition, JNK can phosphorylate the BH3-only protein Bim (Figure 1.12) (Putcha et al, 2003). Under normal conditions, the pro-apoptotic activity of Bim is inhibited by binding to dynein motor complexes. Phosphorylation by JNK releases Bim from these complexes and allows activation of the Bax-dependent apoptotic cascade (Figure 1.14).

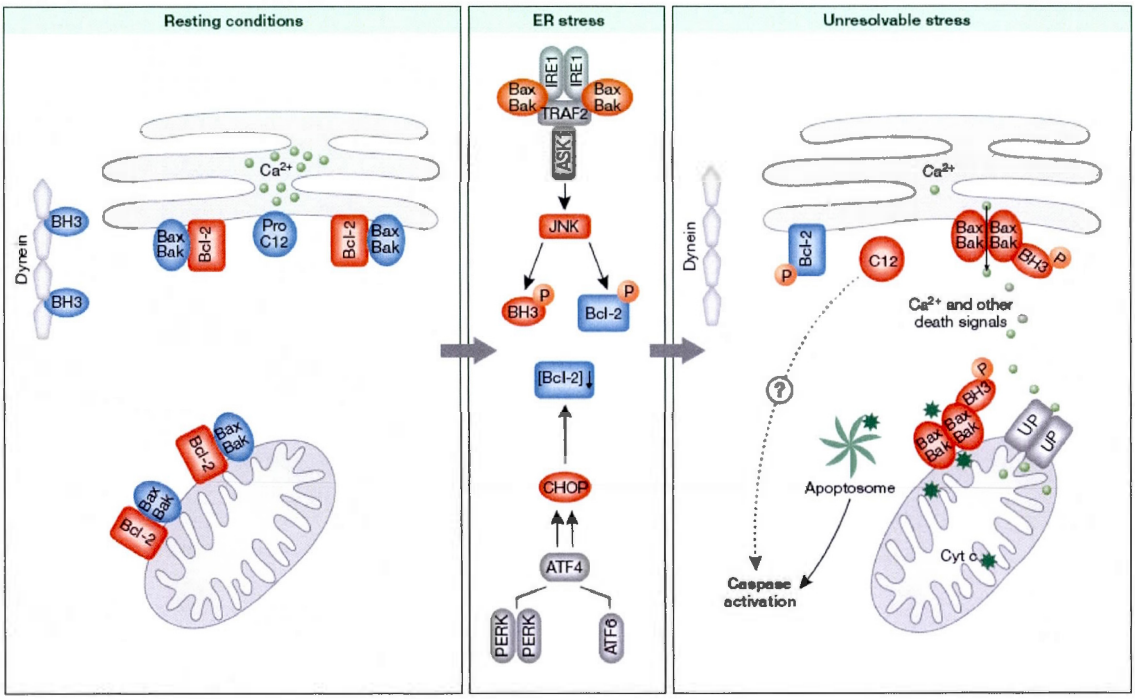


Figure 1.14 Role of Bcl-2 family proteins in ER-mediated apoptosis. Under physiological conditions, the pro-apoptotic proteins Bax and Bak are antagonized by antiapoptotic members such as Bcl-2, both at the mitochondrial and endoplasmic reticulum (ER) membranes. The BH3 only member, Bim is kept inactive by binding to cytoskeletal dynein (BH3 in the figure represents Bim). During ER stress, induction of transcription factor CHOP, and activation of JNK antagonize Bcl-2 and render it inactive by phosphorylation. Activation of JNK also leads to phosphorylation of Bim, leading to its activation. Activated Bim prevents anti-apoptotic Bcl-2 from inhibiting pro-apoptotic Bax and Bak. Consequently, Bax and Bak undergo conformational changes leading to their oligomerization and insertion into the mitochondrial membrane, forming pores and causing release of proteins such as cytochrome c into the cytosol, which contributes to formation of the apoptosome and subsequent activation of the caspase cascade. Figure adapted from Szegezdi et al, 2006.

(3) ER stress can activate pro-apoptotic Bcl-2 family proteins including Bim, Bik and Puma (Boyce and Yuan, 2006; Szegezdi et al, 2009). Bax and Bak co-localize to the ER membrane and undergo oligomerization, which leads to release of ER calcium into the cytoplasm, thus depleting ER calcium stores (Schroder and Kaufman, 2005) (Figure 1.14). The increase in cytosolic levels of calcium activates the protease m-calpain, which can activate caspase-12 (Nakagawa and Yuan, 2000) (Figure 1.13). Thus, Bax and Bak play a role in ER stress-induced apoptosis through regulation of calcium fluxes and amplification of apoptosis through the mitochondrial pathway.

(4) CHOP, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), is a bZIP transcription factor that is induced downstream from the PERK and ATF6 pathways (Oyadomari and Mori, 2004). CHOP sensitizes cells to ER stress-induced apoptosis mainly through down-regulation of Bcl-2, which leads to increased oxidant injury and apoptosis (Malhotra and Kaufman, 2007a) (Figure 1.14). CHOP also induces expression of Bim (Shroder and Kaufman, 2005) and can promote activation and mitochondrial translocation of Bax (Szegezdi et al, 2006). Induction of CHOP appears to perturb the cellular redox equilibrium by causing glutathione depletion and enhanced production of ROS (McCullough et al, 2001). CHOP is also regulated post-translationally by p38 MAPK phosphorylation, which increases its activity (Szegezdi et al, 2006). p38 is a substrate of ASK1, which is recruited to the IRE1-TRAF2 complex during ER stress.

(5) The ER membrane protein B-cell receptor associated protein 31 (BAP31) contains a pseudo-death effector domain and serves as an activation platform for caspase-8 (Ng et al, 1997). After binding and activation, caspase-8 cleaves a 20 kDa fragment (Bap20) from Bap31. This p20 apoptosis inducer fragment causes

calcium-dependent mitochondrial fission; outer mitochondrial membrane permeabilization and cytochrome c release (Nguyen et al, 2000). Bcl-2 and Bcl-xL can bind to BAP31, thus preventing caspase-8 activation, generation of the p20 fragment, and ultimately, transmission of the death signal to mitochondria. Bap31, calnexin and caspase-12 also form a complex that appears to be involved in ER stress-induced apoptosis, through unkown mechanisms (Groenendyk et al, 2006).

A common factor in all three of the major pathways of apoptosis is the tumor suppressor protein p53. DNA damage, induced by stresses such as ROS, can activate p53, which plays an important role in regulation of apoptosis, at both the transcription- and transcription-independent levels.

1.4 THE ROLE OF P53 IN APOPTOSIS

1.4.1 Induction of apoptosis by p53

The role of the tumor suppressor p53 as a transcriptional factor has been extensively described. p53 plays an important role in critical cellular functions such as DNA repair, cell cycle arrest, senescence and apoptosis (Figure 1.15) (Yoshida and Miki, 2010; Schuler and Green, 2001). The p53 gene is one of the most frequent sites for genetic alterations in human solid cancers and is mutated in more than 50% of cancer cases (Hollstein et al, 1991). In unstressed cells, p53 has a short half-life and is maintained at low levels by the protein Mdm2 (Hdm2 in humans) (Figure 1.15). Mdm2 inhibits transcriptional activity of p53 and promotes its degradation by the proteasome (Haupt et al, 1997).

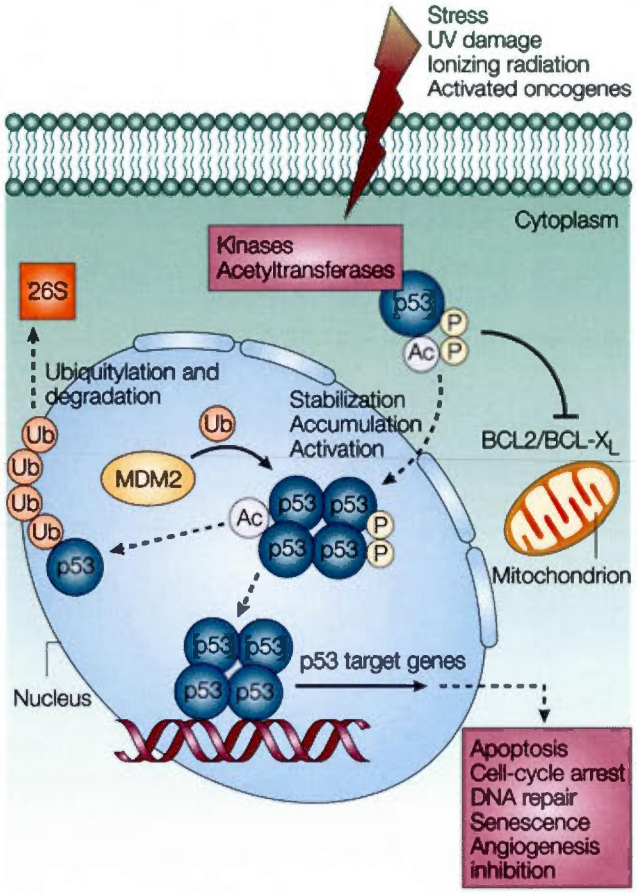


Figure 1.15 Activation of p53 and cellular response. Activation of cellular stress pathways leads to post-translational modifications and tetramerization of p53, stabilizing the protein and leading to its accumulation in the nucleus. Transcriptionally, p53 induces the activation of its target genes that are implicated in various cellular functions such as apoptosis, cell-cycle arrest or DNA repair. When p53 is no longer needed, it is targeted for ubiquitylation by Mdm2 and moved out of the nucleus to be degraded by the 26S proteasome. Figure adapted from Bode et al, 2004.

p53 protein levels increase rapidly in response to different stress signals, and when the genomic integrity of a cell is threatened by metabolic or genetic disorders. These factors include DNA damage, ROS, nucleotide depletion, hypoxia and oncogene activation (Liu et al, 2008). p53 becomes stabilized and transformed into an active form, mainly due to post-translational modifications including phosphorylation, acetylation and methylation. DNA damage causes p53 stabilization through activation of the Ataxia-telangiectasia mutated kinase (ATM)/checkpoint kinase 2 (Chk2) pathway. Under normal circumstances Chk2 is kept inactive by binding to protein phosphatase 2 (PP2A). Phosphorylation of Chk2 by ATM results in its dissociation from PP2A leading to its activation. The fully active Chk2 in turn phosphorylates and stabilizes p53. The activation of these upstream kinases is opposed by an inhibitory phosphatase, Wip 1, which is induced by stabilized p53 (Lu et al, 2008). This mode of negative feedback functions in addition to that provided by the ubiquitin ligase Mdm2. Thus, these feedback loops maintain low levels of p53 in undamaged cells, and can attenuate signals once lesions are repaired. Thus, modulated DNA damage signals cause the cyclical activation and deactivation of p53 (Batchelor et al, 2008) (Figure 1.16).

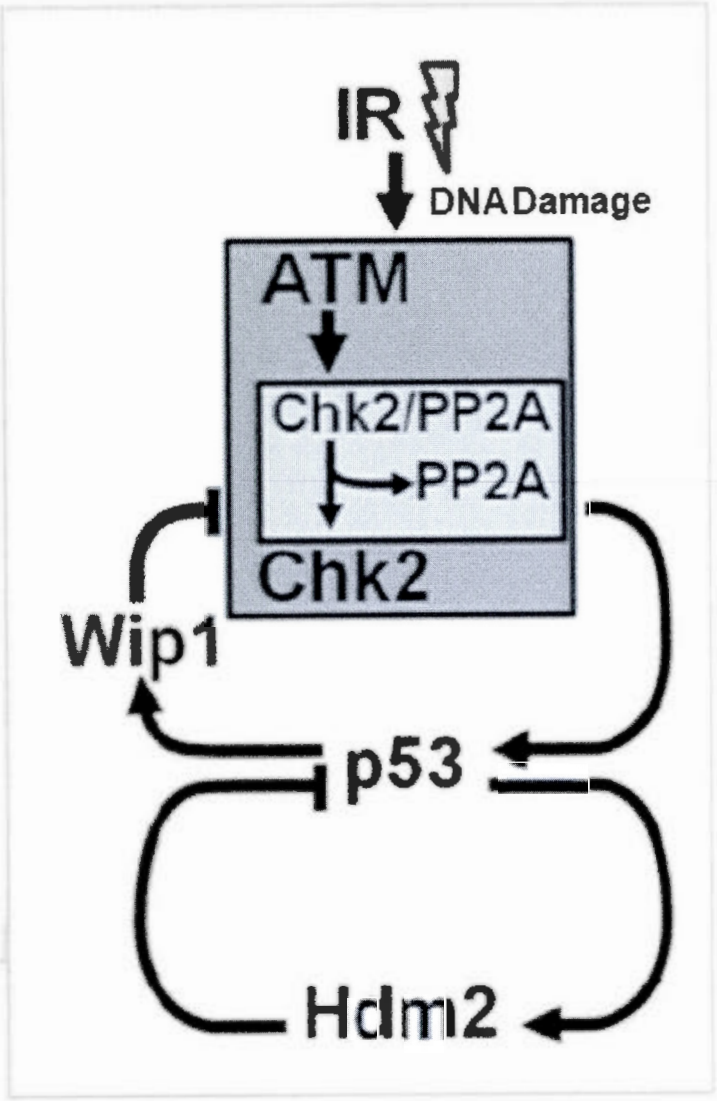


Figure 1.16 Regulation of p53. Mdm2 (human analogue, Hdm2) negatively regulates p53 through ubiquitin-mediated proteolysis that subsequently degrades p53. DNA damage caused by ionizing radiation leads to activation of the ATM-Chk2 pathway. Upstream signals lead to dissociation of p53 from the ubiquitin ligase Hdm2 and thereby p53 becomes stabilized. The ATM-Chk2 pathway is in turn regulated by the protein phosphatase, Wip 1, which is induced by stabilized p53. Together, these feedback circuits keep levels of p53 low in undamaged cells, and can attenuate signals once lesions are repaired. Figure adapted from Freeman et al, 2010 (modified).

Cell cycle arrest and apoptosis are two of the most studied p53-mediated cellular responses that reflect the choice of p53 to determine a cell's ultimate fate, life or death (Yoshida and Miki, 2010). However, the signals that determine cell fate remain unclear.

1.4.2 Choice of response-Life or Death: The two faces of p53

The decision between life and death is dependent on the balance between the availability of cellular factors as well as extracellular signaling events. The type of stress versus the extent of damage that triggers p53 activation is an important factor, which indicates whether or not the injury is worth repairing. If damage is not too severe, p53 acts by causing cell cycle arrest to allow the cell to repair the damage, before continuing through the cell cycle. If damage is too extensive, then the damaged cell is eliminated by apoptosis to maintain genomic fidelity. Thus, p53 is known as the <<guardian of the genome>> (Lane, 1992). There are numerous targets of p53 that participate in different cellular processes and the actions of these targets ultimately decide the fate of a cell subjected to stress.

1.4.3 Role of p53 in apoptosis

At the transcriptional level, phosphorylated p53 induces transcription of pro-apoptotic proteins such as Bax, Bid, Puma and Noxa, and it suppresses the activity of anti-apoptotic proteins such as Bcl-2 and IAPs (Figure 1.17) (Schuler and Green, 2001; Yoshida and Miki, 2010; Miyashita and Reed, 1995; Yu and Zhang, 2008). Puma can antagonize mitochondrial Bcl-2 proteins, thus promoting mitochondrial translocation and multimerization of Bax (Yu and Zhang, 2008). Noxa is induced by p53 in response to X-ray irradiation (Oda et al, 2000). It contributes to p53-mediated apoptosis in a similar manner to Puma and Bax. Other apoptotic proteins induced by p53 include p53-inducible genes (PIG), p53-induced protein with death domain (PIDD), p53-apoptosis effector related to PMP-22 (PERP), Scotin, and p53-regulated apoptosis-inducing protein 1 (p53AIP1). The p53 target, p53AIPI can regulate mitochondrial membrane potential and

trigger the release of Cyt c by interacting with Bcl-2 (Matsuda et al, 2002). Many of the PIG genes encode for redox active proteins, including two ROS-generating enzymes, human NAD(P)H:quinone oxidoreductase (NQO1) and proline oxidase (POX, PIG6) (Liu et al, 2008). In the extrinsic pathway, p53 induces expression of the death receptors Fas and DR5, along with the death ligands FasL and TRAIL (Schuler and Green, 2001; Yoshida and Miki, 2010) (Figure 1.17). p53 may also induce apoptosis via an ER-dependent mechanism by transactivating the expression of Scotin, a protein located in the ER and at the nuclear membrane (Bourdon et al, 2002).

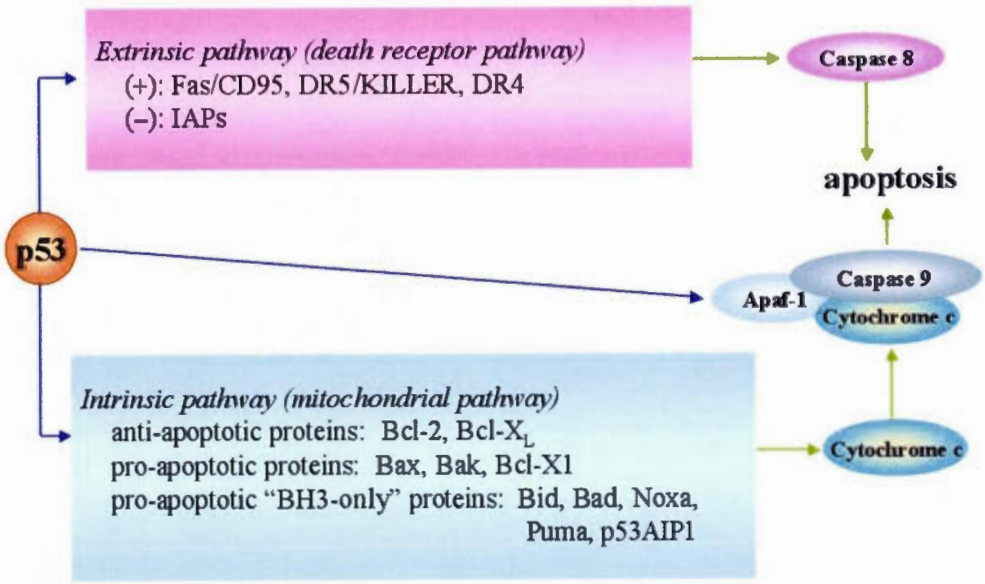


Figure 1.17 p53-apoptotic pathways. In response to cellular stress, p53 transactivates pro-apoptotic genes that activate the (1) death receptor pathway (CD95/Fas, Bid) and (2) the mitochondrial pathway (Bax, Noxa, Puma). Figure adapted from Bai et al, 2006.

Further to its activity at the transcriptional level, p53 protein can also promote apoptosis through several transcription-independent mechanisms (Vaseva and Moll, 2009). The first mechanism established that p53 rapidly translocates to the mitochondrial

membrane. Wild type p53 is able to interact physically and functionally with anti-apoptotic proteins such as Bcl-2, Bcl-X_L and Mcl-1 at the mitochondrial membrane, to displace previously sequestered pro-apoptotic proteins such as Bax, Bak and t-Bid. p53 is able to displace t-Bid and Bax from inhibitory complexes formed with Bcl-X_L (Chipuk et al, 2004). Furthermore, p53 interacts with Bak and liberates it from its inhibitory complex with Mcl-1 (Vaseva and Moll, 2009). p53 can also induce Bax and Bak oligomerization, which facilitates cytochrome c release after mitochondrial membrane permeabilization (Mihara et al, 2003; Chipuk et al, 2004).

In a parallel transcription-independent pathway, p53 protein is sequestered in the cytoplasm in an inactive complex by Bcl-X_L in non-stressed cells (Chipuk et al, 2005) (Figure 1.18). During genotoxic stress, nuclear p53 induces Puma, which liberates cytoplasmic p53 from Bcl-X_L, thus allowing p53 to translocate to mitochondria and activate Bax, thus directly inducing apoptosis in a transcription-independent manner. Puma can therefore couple the nuclear and cytoplasmic apoptotic functions of p53.

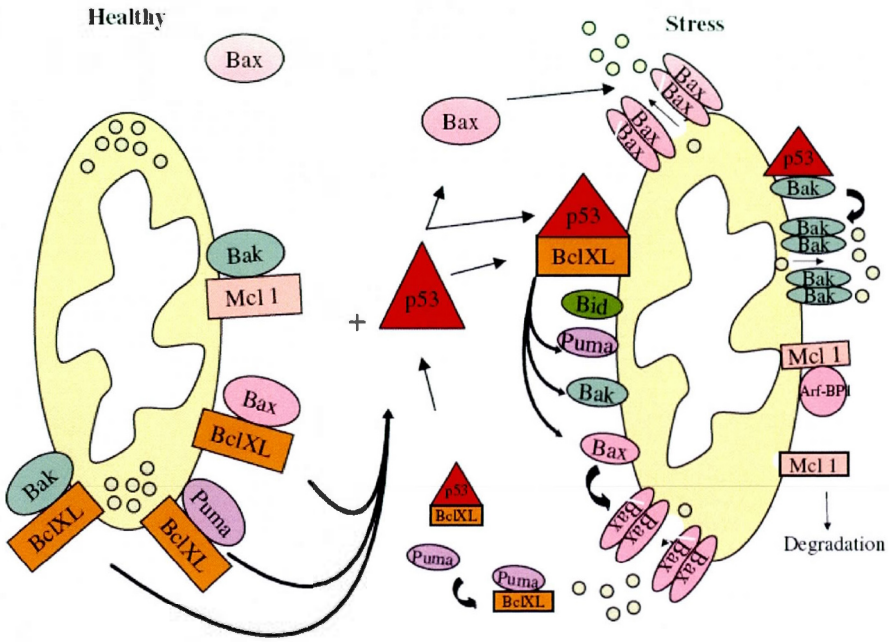


Figure 1.18 p53 and Bcl-2 family proteins at the level of mitochondria. p53 protein is sequestered in the cytoplasm in an inactive complex by Bcl-X_L in non-stressed cells. Activation of p53 by stress induces Puma, which liberates cytoplasmic p53 from Bcl-X_L, thus allowing p53 to translocate to mitochondria and activate Bax, thus directly inducing apoptosis in a transcription-independent manner. Figure adapted from Moll et al, 2006.

Genotoxic stress can result in p53-mediated activation of caspase-2. The binding of p53 to death domain-containing adaptor proteins, PIDD and RAIDD leads to recruitment of procaspase-2. The resulting ternary complex leads to caspase-2 activation. Activated caspase-2 can trigger Bid-mediated activation of the mitochondrial pathway of apoptosis (Lavrik et al, 2006) (Figure 1.19). Thus, tumor suppressor p53 can regulate apoptosis both transcription-dependent and –independent mechanisms. Mutations in the DNA-binding site of p53 in human tumors impairs both the transcriptional and mitochondria pro-apoptotic functions of p53 (Mihara et al, 2003).

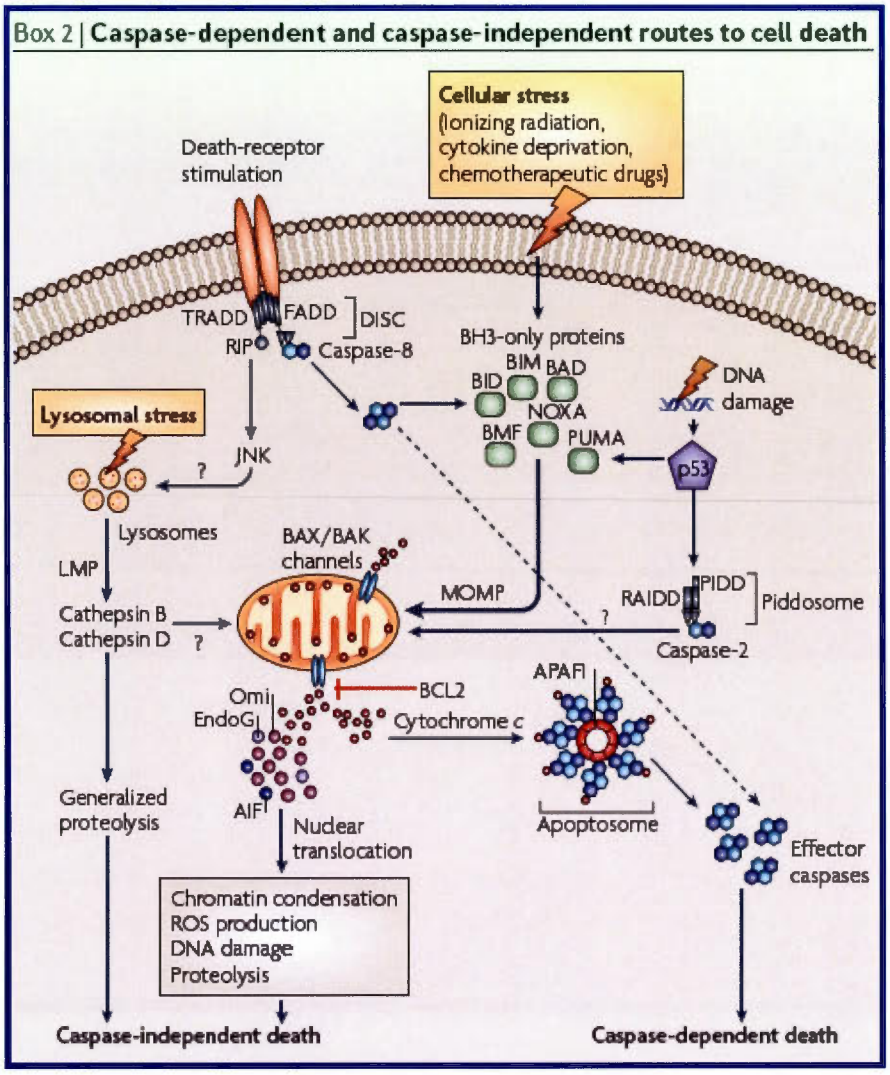


Figure 1.19 p53-associated pathways involved in apoptotic cell death. In response to DNA damage, p53 activates cell death by up-regulating BH3-only proteins such as PUMA and NOXA, which promotes Bax or Bak activation, leading to mitochondrial outer membrane permeabilization (MOMP), cyt c release, apoptosome formation, caspase-9 activation, and the caspase-dependent cascade. In an alternative pathway, caspase-2 acts upstream of MOMP and is possibly activated by different complexes, such as the PIDDosome or DISC (through caspase-8). Once activated caspase-2 cleaves and activates Bid, which causes MOMP and cyt c release. Figure adapted from Maiuri et al., 2007.

1.4.4 p53 and oxidative stress

A complex relationship exists between p53 and levels of ROS. Low levels of DNA damage caused by oxidative stress can be repaired, whereas extensive damage usually results in cell death. p53 contributes to both outcomes by stimulating expression of either pro- or antioxidant genes (Figure 1.20).

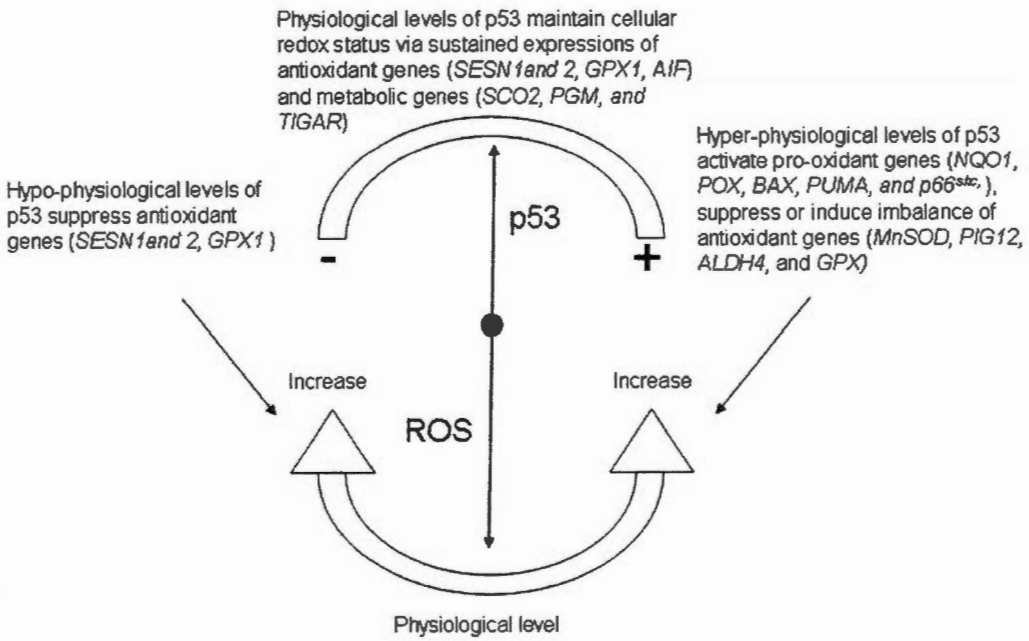


Figure 1.20 Levels of ROS regulate p53 function. Figure adapted from Liu et al, 2009.

During extensive cellular stress, p53 levels increase and induce the expression of pro-oxidant genes, which elevate ROS levels, leading to cell death by apoptosis. Under normal conditions, levels of ROS are low and cells experience only mild oxidative stress. In such situations, p53 enhances cell protective mechanisms by up-regulating the expression of several antioxidant genes, including Gpx, MnSOD, and mammalian sestrin

homologs SESN1 and SESN2, which are involved in the regeneration of oxidized peroxiredoxins (Figure 1.20). This differential control of redox levels within a cell is attributed to the levels of p53 itself. Thus, low levels of p53 suppress ROS within the cell, while higher levels of p53 promote ROS accumulation (Liu et al, 2008).

1.5 PROTECTIVE/ADAPTIVE MECHANISMS AGAINST STRESS-INDUCED APOPTOSIS

Cellular stress signals can contribute to evasion of apoptosis by activating a group of powerful anti-apoptotic genes. This includes activation/up-regulation of mitogenic signaling pathways (e.g. Akt pathway), anti-apoptotic proteins (IAPs, FLIP, Bcl-2), ROS scavengers (antioxidants), and heat shock proteins (Hsps) (Figure 1.21).

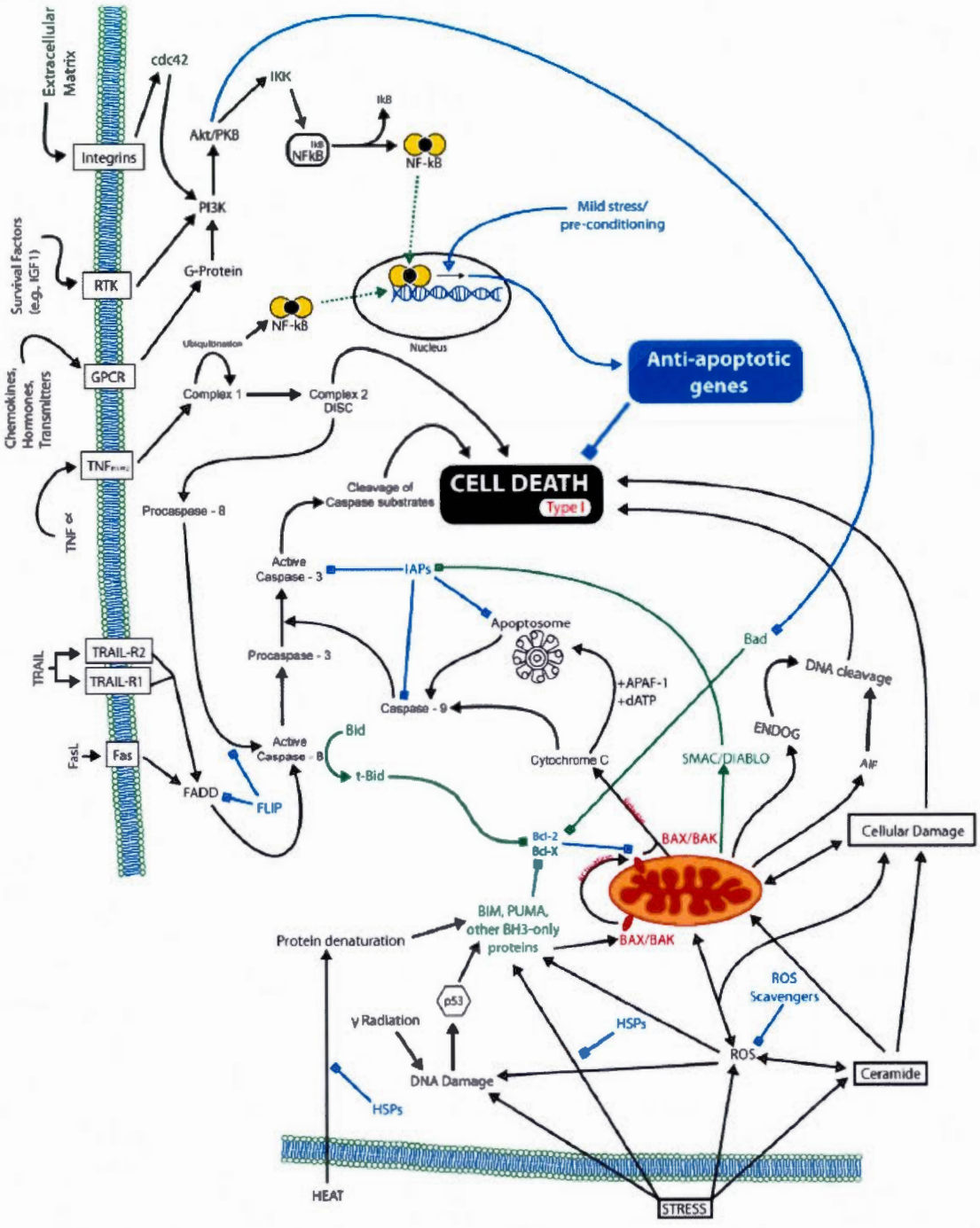


Figure 1.21 Figure depicting the role of anti-apoptotic proteins (Akt, IAPs, FLIP, Bcl-2, Bcl-X_L, ROS scavengers and Hsps) in the regulation of major pathways of apoptosis. Figure adapted from Portt et al, 2011.

1.5.1 The serine/threonine protein kinase Akt

Growth factor stimulation of phosphoinositide 3-kinase (PI3K) activity leads to Akt activation. The mechanism by which Akt protects cells from death is likely to be multifactorial, because Akt directly phosphorylates several components of the cell- death machinery. The pro-apoptotic protein, Bad is one of the targets of Akt activation. In the unphosphorylated state, Bad is targeted to the mitochondria where it forms a complex with Bcl-2 or $BclX_L$, inhibiting their anti-apoptotic activity. Phosphorylation of Bad by Akt inhibits its pro-apoptotic effect (Figure 1.21) (Datta et al, 1997; Harvey and Lonial, 2007). Akt also regulates cell death by inhibiting caspase-9 activation by phosphorylation (Cardone et al, 1998). Akt can also activate the transcription of anti-apoptotic genes through activation of the transcription factor NFκB (Figure 1.21). NFκB is kept inactive in the cytoplasm by binding to its inhibitor IκB. Akt can phosphorylate and activate IκB kinase (IKK), a kinase that induces proteosomal degradation of IκB. This allows NFκB to translocate to the nucleus and activate transcription of a variety of substrates including anti-apoptotic genes such as c-IAP1 and 2 (Gagnon et al, 2003; Chen et al, 2006).

1.5.2 Inhibitor of Apoptosis Proteins (IAPs)

Caspase activity can be regulated by a family of proteins known as IAPs (Figure 1.21). These proteins bind to procaspases under physiological conditions and thus prevent their activation. In mammals, the members of the IAP family include X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein 1, 2 (cIAP-1, cIAP-2), neuronal apoptosis inhibitory protein (NAIP), and survivin. These proteins are characterized by the presence of 1-3 baculoviral IAP repeats (BIR) (Figure 1.22) (Altieri, 2010).

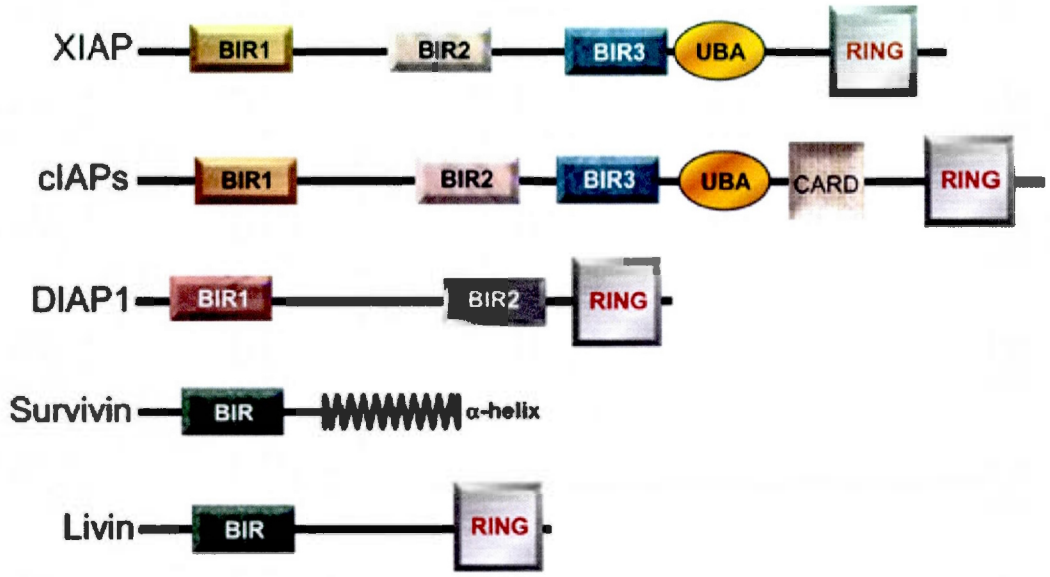


Figure 1.22 Schematic representation of domain structure in representative IAPs. The presence of at least one BIR domain is the defining characteristic of the IAP family. Along with the BIR domains, several IAPs contain a RING-zinc finger domain at the carboxy terminus. c-IAPs (1/2) both possess a caspase recruitment domain (CARD) in the linker region between the BIR domains and the RING domain. Figure adapted from Altieri, 2010.

IAPs function as direct inhibitors of both initiator and effector caspases. IAPs such as XIAP, cIAP-1 and c-IAP-2 were shown to bind directly to caspase-3, -7 and -9 and inhibit their activity (Shiozaki et al, 2003; Scott et al, 2005). In addition, cIAP-1 and cIAP-2 can bind to TRAF2 by means of their BIR domain and thus negatively regulate death receptor-mediated apoptosis by promoting the activation of cell survival factor NF-κB (Chu et al, 1997; Hunter et al, 2007). The activity of IAPs in turn is regulated by mitochondrial pro-apoptotic proteins, SMAC/DIABLO, and HtrA2/Omi (Figure 1.21).

1.5.3 FLIP

The death receptors (DISC) - mediated apoptotic pathway can be regulated by FLICE-like inhibitory protein (c-FLIP) (Figure 1.21). It is a cytosolic caspase-8/FLICE inhibitor, which exists in two different forms: long form (c-$FLIP_L$) and short form (c-$FLIP_s$). Because of its sequence homology to caspase-8, it competes with caspase-8 for binding to the death effector domain (DED) of adaptor protein FADD. The interaction of c-FLIP with FADD prevents DISC-mediated processing and activation of caspase-8 (Zimmermann et al, 2001; Ghavami et al, 2009).

Thus, IAPs and FLIP play an important role in regulation of caspase-dependent apoptosis (Figure 1.21).

1.5.4 Bcl-2 Family proteins

The mitochondrial pathway of apoptosis is regulated by a balance between the pro-apoptotic and anti-apoptotic members of the B-cell lymphoma protein 2 (Bcl-2) super families of proteins. The anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-w, A1 and Mcl-1) and proapoptotic (Bax, Bak, Bad, Bim, Bid, Bik, Bmf, Hrk, p53-upregulated modulator of apoptosis (Puma) and Noxa) proteins play a major role in mitochondrial outer membrane permeabilization and susceptibility to apoptosis (Youle and Strasser, 2008). Proteins such as Bad, Bik, Bid, Bim, Hrk, Puma and Noxa have a conserved Bcl-2 homology domain 3 (BH3) that can bind and regulate the anti-apoptotic Bcl-2 proteins, thus promoting apoptosis. The pro-apoptotic member Bak and the anti-apoptotic members Bcl-2 and Bcl-X_L are integral membrane proteins that are present mainly in the outer mitochondrial membrane. The anti-apoptotic Bcl-2 family members (Bcl-2 and Bcl-X_L) inhibit apoptosis by binding Bax and Bak (Figure 1.21), sequestering truncated Bid (t-Bid) and Apaf-1, and thus preventing the release of proteins such as cytochrome c and Smac/Diablo from mitochondria (Figure 1.23).

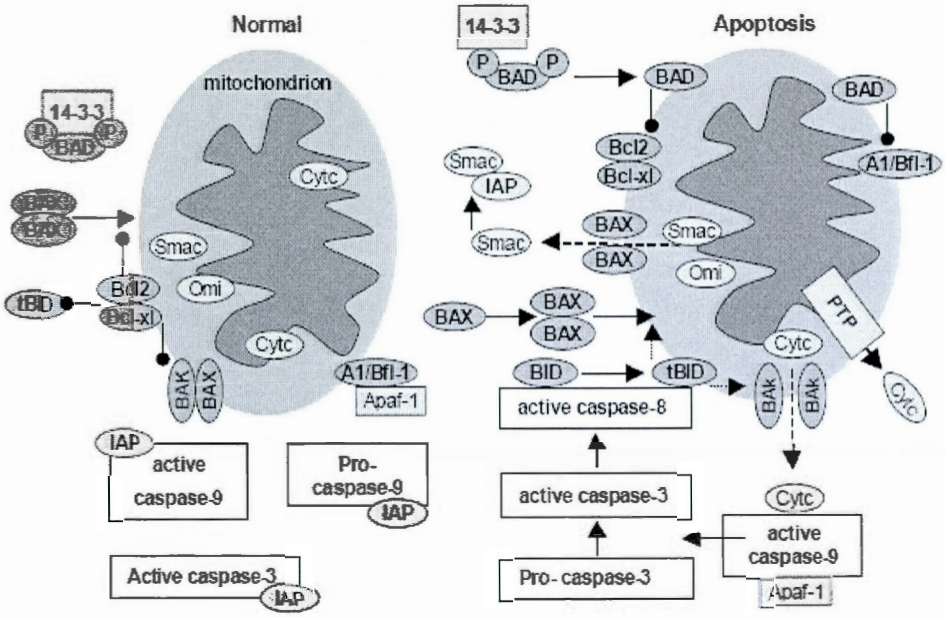


Figure 1.23 Diagram depicting the state of Bcl-2 family proteins during normal and apoptotic conditions. Under normal conditions, pro-apoptotic proteins (Bax, Bak, and Bid) are kept inactive by the anti-apoptotic proteins Bcl-2 and Bcl-X_L. During an apoptotic stimulus, the activation of BH3-only proteins (e.g. Bad) antagonizes anti-apoptotic proteins Bcl-2 and Bcl-X_L. This allows the activation of Bax and Bak, release of cyt c, activation of caspase-9 and caspase-3, and execution of apoptosis. Figure adapted from Schoemaker and Moshage 2004.

Under normal conditions, pro-apoptotic proteins such as Bax, Bid and Bad are located in the cytosol. Upon an apoptotic stimulus, Bid can be cleaved by caspase-8, -10, or -2 to form t-Bid, whereas Bax undergoes oligomerization. Bad is released from the adaptor molecule 14-3-3 by dephosphorylation (Figure 1.23). These events lead to translocation of t-Bid, Bax and Bad to the outer mitochondrial membrane. At the mitochondrial membrane, Bad interacts with and antagonizes the anti-apoptotic proteins Bcl-2 and/or Bcl-X_L (Figure 1.21), whereas Bak and Bax require an interaction with t-Bid to form pores in the mitochondrial membrane, which allow the release of cytochrome c. The pro-apoptotic proteins Puma and Noxa are induced by p53 in response to DNA damage

(Youle and Strasser, 2008). Puma mainly acts indirectly by activating Bax and/or Bak, and by blocking the inhibition of these proteins by anti-apoptotic proteins such as Bcl-2, Bcl-X_L (Figure 1.21) (Yu and Zhang, 2008). It appears that Puma can also trigger apoptosis by directly activating Bax.

Thus, mitochondrial anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-X_L protect the mitochondria by inhibiting Bax activation and its subsequent mitochondrial translocation (Chao et al, 1995), thus preventing Bax from disrupting outer mitochondrial membrane integrity. Therefore, the release of cytochrome c and subsequent pro-caspase activation are inhibited, favoring cell survival (Yang et al, 1997).

1.5.5 Thermotolerance (heat pre-conditioning)

Thermotolerance can be defined as the ability of the body and its cellular structures to withstand severe destructive heat stress. Exposure of cells to transient, non-lethal elevations in temperature activates cellular stress reponses and induces a state of thermotolerance, which renders the cells resistant to subsequent lethal insults such as those induced by heat shock, oxidative stress, chemotherapeutic agents, radiation and environmental stressors (Ritcher et al, 2010; Martindale et al, 2002; Kregel et al, 2002). Thermotolerance can be developed following shorter exposures (e.g. 30 min) to lethal temperatures (42-45 ^{o}C) or during continous heating (e.g. 3-24 h) at non-lethal temperatures (39.5-41.5 ^{o}C) (Landry et al, 1982; Przybytkowski et al, 1986). It is a transient process and usually declines within several days. The adaptive role of mild thermotolerance against apoptotic pathways is relatively unknown in humans. It is associated with the synthesis and cellular accumulation of a family of highly conserved proteins referred to as heat shock proteins (Hsps) (Figure 1.21), which bind to various molecules to provide cell protection (Review Ritcher et al, 2010).

1.5.5.1 Heat shock proteins

Hsps, also called stress proteins, are synthesized by all organisms, not only in response to increased temperatures but also to a diverse array of stresses, such as oxidative stress, toxic metals, anoxia and radiation. Increased gene transcription occurs in response to all conditions that contribute to denatured or abnormally folded proteins. These conditions can result from environmental stress, which includes exposure of cells to heavy metals, amino acid analogues, or oxidative stress (Scharf et al, 1998) or pathophysiological stress, including pathogen infection, inflammation or fever (Morimoto, 2008). Non-stressful events or components associated with increased expression of Hsps include development and differentiation of cells, growth factors and oncogenes, or prooncogenes (Georgopoulis and Welch, 1993) (Figure 1.24).

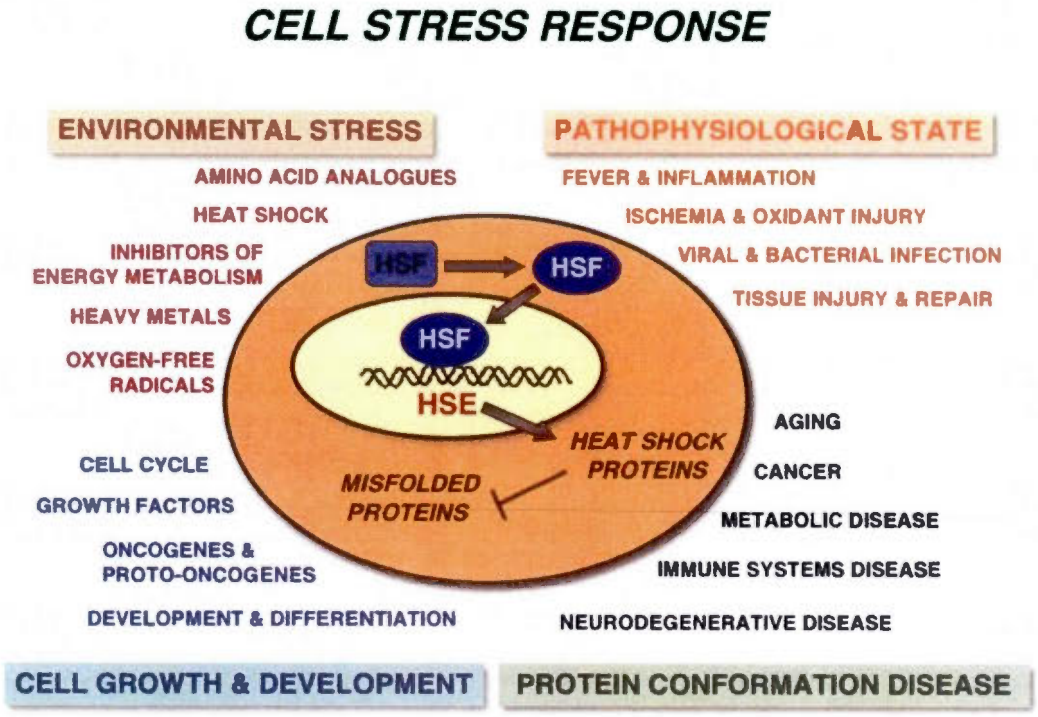


Figure 1.24 Diagram illustrating different stress conditions that induce the heat shock response. (Figure adapted from Morimoto 2008).

A set of transcription factors, known as heat shock factors (HSF), are responsible for the induction of protective genes which in turn induce the expression of the Hsps (Pirkkala et al, 2001; Shabtay et al, 2006). HSF1 is implicated in the heat shock response and developmental process because mice lacking HSF1 were unable to develop thermotolerance or induce heat responsive genes upon heat shock (Mcmillan et al, 1998; Zhang et al, 2002), confirming that HSF1 in particular is responsible for the heat shock response.

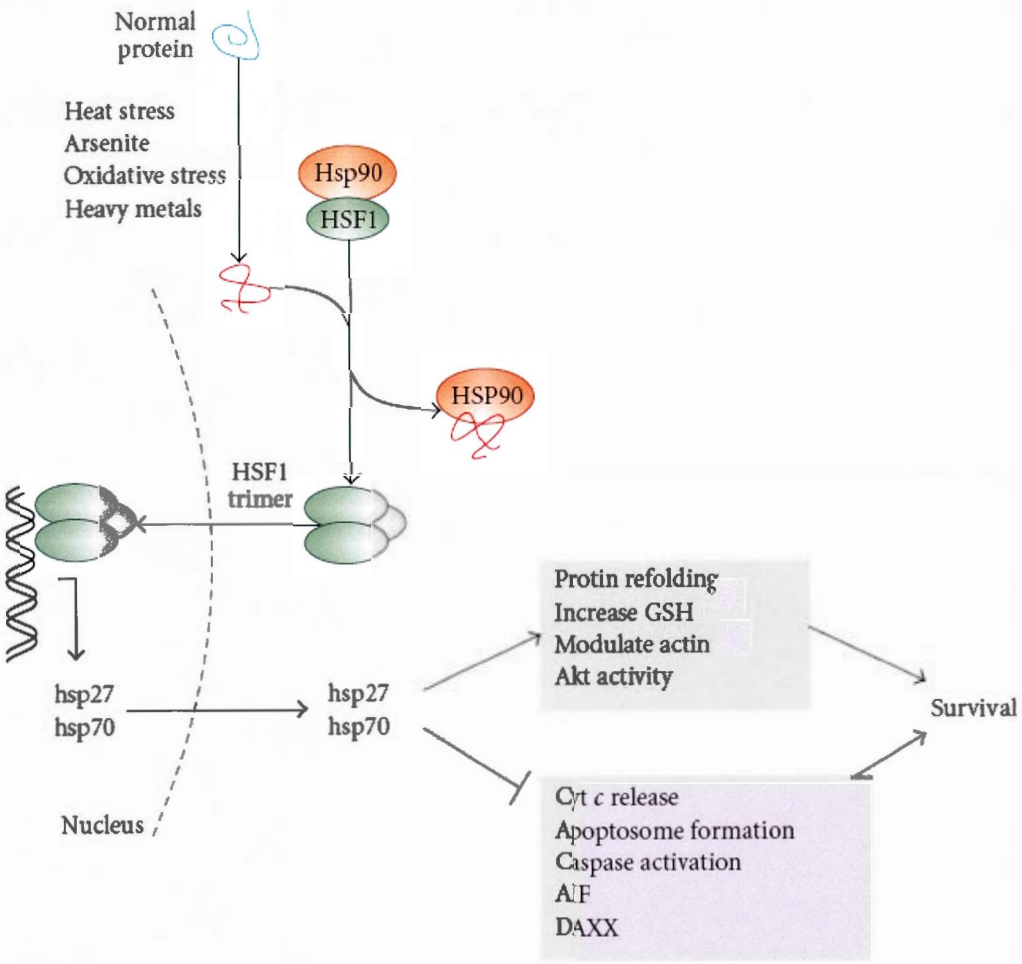


Figure 1.25 Induction of Hsps inhibits apoptosis and promotes cell survival. Stress-inducing agents, such as heat, oxidants, toxins and heavy metals, initiate trimerization of HSF-1 monomers in the cytoplasm. These trimers translocate into the nucleus and induce the transcription of Hsp genes such as Hsp27 and Hsp70. These Hsps regulate various events of the apoptotic pathways and promote cell survival. Figure adapted from Fulda et al, 2010.

Inactive HSF-1 is maintained in a monomeric form through interaction with Hsp90 and cochaperones in the cytosol (Figure 1.25). During stress, accumulation of unfolded proteins competes with HSF1 for binding to Hsp90. Thus, release of HSF1 from the complex results in its transition from a monomer to a homotrimer, which translocates to the nucleus and binds to DNA, leading to the expression of heat shock proteins (Figure 1.25). These proteins play a central role in the development of thermotolerance.

Hsps are generally classified into two groups according to their size: high and low molecular weight Hsps. The larger Hsps are subdivided into three major families, Hsp60, Hsp70 and Hsp90, which function as ATP-dependent chaperones. The smaller Hsp group is comprised mainly of the ATP-independent chaperone Hsp27 (Sreedhar and Csermely, 2004). Many members of these Hsp families are present constitutively in cells and are involved in the regulation of normal homeostasis. However, some Hsps are expressed only after stress. These inducible Hsps help cells to cope and recover from the stressful situation. Among the different Hsps, Hsp27 and Hsp70 are the most strongly induced after stresses such as anticancer drugs, oxidative stress and irradiation .The inducible as well as the constitutively expressed members of the Hsp families, are known as molecular chaperones, which i) help in normal folding of various polypeptides, ii) assist mis-folded proteins to attain or regain their native states, iii) regulate protein degradation and/or iv) help in translocation of proteins to different cellular compartments (reviewed in Ritcher et al, 2010; Hartl and Hayer-Hartl 2002). The above functions imply that these proteins interact with a large variety of cellular proteins and thus are important components of cellular networks. The cytoprotective function of these proteins is mainly explained by their anti-apoptotic function.

1.3.2.4 Hsps: Role in apoptosis

Data accumulated in the last 10 years clearly demonstrate that these proteins have essential anti-apoptotic properties. They can combine with key stress signalling and apoptotic molecules, thereby blocking cell death and promoting survival, proliferation or differentiation. These proteins can block the apoptotic pathways (both intrinsic and extrinsic) through the interaction with key proteins at three levels: 1) upstream of the mitochondria, thereby modulating signaling pathways; 2) at the mitochondrial level, controlling the release of apoptogenic molecules; 3) and at the post-mitochondrial level.

Studies show that increased expression of Hsp27, Hsp70 or Hsp90 can protect against apoptosis induced by different stimuli, including hyperthermia, oxidative stress, ligation of the Fas death receptor, staurosporine, or anti-cancer drugs (Garrido et al, 1997; Mehlen et al, 1996). The most important anti-apoptotic role of Hsp27 involves its ability to reduce cellular ROS levels by enhancing the antioxidant defense of the cell (Preville et al, 1999). Thus, Hsp27 regulates both redox homeostasis and mitochondrial stability of the cell. Also, Hsp27 can bind to both cytochrome c and procaspase-3 in the cytoplasm, thus preventing apoptotic events taking place downstream of the mitochondria (Concannon et al, 2001). Hsp27 can interact with DAXX and also regulate caspase-independent apoptosis (Charrette et al, 2000).

Hsp70 has been shown to inhibit the apoptotic pathways at different levels (Figure 1.26). At the mitochondrial level, it inhibits Bax translocation and insertion into the outer mitochondrial membrane, thus preventing mitochondrial cytochrome c release. At the post-mitochondrial level, it can interact with Apaf-1, thereby preventing the recruitment of procaspase-9 to the apoptosome and subsequent activation of caspase-9 (Beere et al, 2000). Hsp70 was also shown to regulate caspase-independent cell death by interacting with AIF, which neutralizes its DNase activity (Ravagnan et al, 2001; Gurbuxani et al, 2003). Hsp70 inhibits TNF-induced cytochrome c release by suppressing caspase-8-

mediated cleavage of Bid and its activation (Gabai et al, 2002; Paul et al, 2002). A very recent study showed that Hsp72 protects cells from ER stress-induced apoptosis via enhancement of IRE1α-XBP-1 signaling (Gupta et al, 2010).

Hsp90 can interfere with apoptosome formation by sequestering Apaf-1 and thereby preventing caspase-9 activation (Pandey et al, 2000). Hsp90 can bind directly with Akt and protect it from protein phosphatase 2A (PP2A)-mediated dephosphorylation. This enables phosphorylated Akt to phosphorylate Bad, thereby leading to caspase-9 inactivation and cell survival (Basso et al, 2002; Cardone et al, 1998; Datta et al, 1997).

Therefore, Hsps play a major role in the regulation of the three major pathways of apoptosis, but it is still unclear whether the anti-apoptotic influence of thermotolerance is caused by Hsps alone or if other defense proteins such as anti-oxidants and anti-apoptotic proteins are also implicated in the adaptive survival role of this phenomenon.

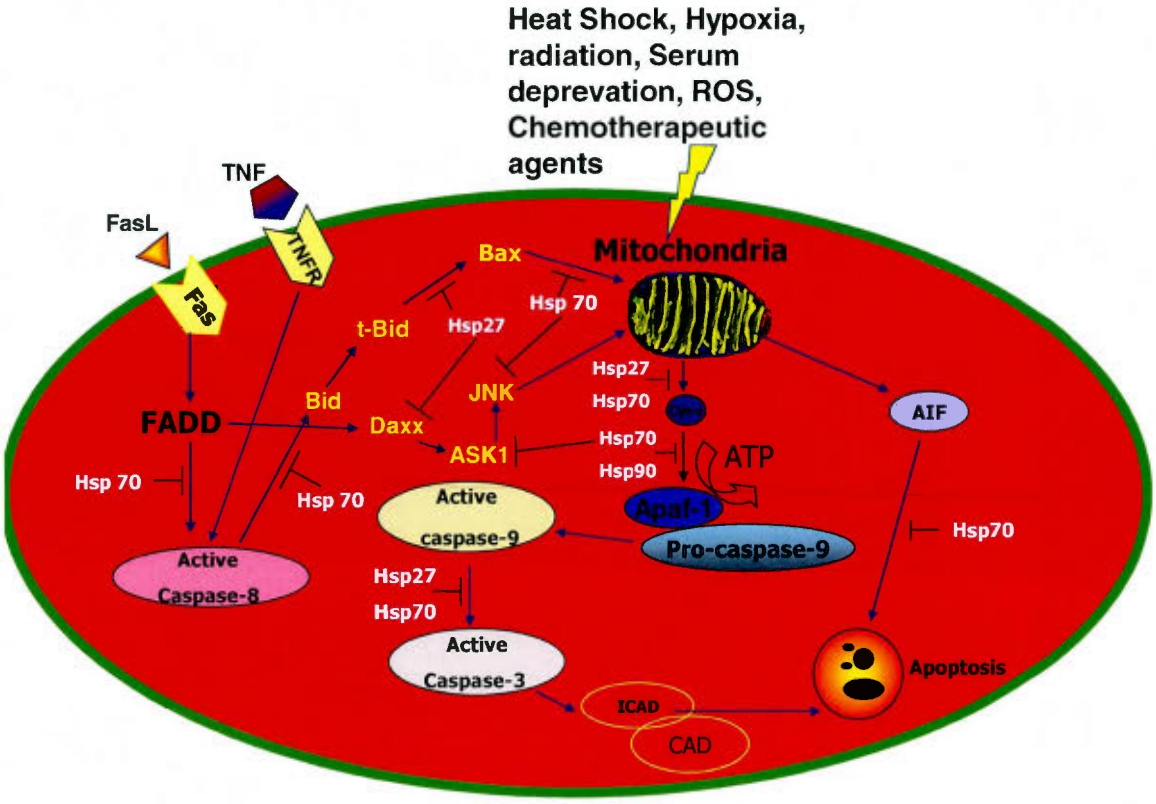


Figure 1.26 Events regulated by Hsps in the intrinsic (mitochondrial) and extrinsic (death receptor) pathways of apoptosis. Hsps intervene at multiple points both upstream and downstream of mitochondria. Hsp27 inhibits apoptosis by preventing the mitochondrial release of cytochrome c into the cytoplas, as well as by sequestering procaspase-3 in the cytosol, thus preventing its activation. Hsp70 regulates death receptor induced apoptosis by preventing recruitment of procaspase-8 to the death complex. It also prevents the cleavage and activation of Bid. It further acts downstream by preventing cytochrome c release into the cytosol and sequestering Apaf-1, inhibiting apoptosome formation, as well as activation of caspase-3 by sequestering cytosolic procaspase-3. Hsp70 can also regulate caspase-independent cell death through suppression of AIF activity. Hsp90 sequesters Apaf-1 and thereby prevents apoptosome formation. Hsp27 and Hsp70 can regulate stress mediated by the JNK pathway, by inhibiting the recruitment of DAXX and ASK1 to the Fas signalling complex.

1.6 PRESENTATION OF PROJECT

Cancer is one of the major diseases affecting people all over the world. Several treatment strategies (e.g. chemotherapy, radiotherapy, immunotherapy and surgery) were developed for treating cancer. Hyperthermia is one of the most innovative methods of treating cancer, mainly used as an adjuvant to radiotherapy and chemotherapy. The scientific basis for the use of hyperthermia as an adjunct for the treatment of cancer rests on four main observations:

1) Tumor cells are more sensitive to the effects of increased temperature (42-45 °C) compared to the normal cells.
2) The combination of chemotherapeutic drugs and hyperthermia produces additive and synergetic killing effects on tumor cells. These effects appear more dramatic at low pH.
3) Hyperthermia produces changes in the tumor blood flow and oxygen.
4) Hyperthermia augments the immune responses of the patient toward tumors. These would include effects on macrophages, natural killer (NK) cells, and T-cell cytotoxic effector functions.

Hyperthermia has the potential to increase cytotoxic effects of radiation or chemotherapeutic agents within the tumor volume, without increasing normal tissue toxicity. In the clinic, this technique is applied in combination with radiation and cytotoxic agents by localized heating of the tumor region at higher temperatures such as 42-43°C for 1-2 h or by milder heating of the body at 39.5-41°C for longer times.

An exposure to mild heat shock induces the development of thermotolerance, which is accompanied by the transcriptional activation and accumulation of a group of highly conserved proteins called heat shock proteins (Hsps). These proteins can protect cells against damage caused by different stresses by preventing protein denaturation and by repairing denatured proteins in order to restore their biological activity. The heat shock

response has been largely studied at relatively high, lethal temperatures such as 42 to 45°C. However, the heat shock response at low non-lethal temperatures such as 39-40°C within the physiological fever range has received little attention and is not clearly understood.

Cancer patients undergoing high dose chemotherapy often experience recurrent fevers during periods of neutrophil deprevation. It has not been determined in vivo whether these febrile temperatures could induce thermotolerance and Hsps, which could attenuate tumor responses to cytotoxic treatments such as chemotherapy, and radiotherapy, which aim to destroy tumor cells by apoptotic cell death.

1.6.1 Hypothesis

The hypothesis is that "Mild thermotolerance induced at non-lethal temperatures (e.g. 39-40°C) is an adaptive physiological response that can increase cellular defenses and protect cells against different stresses such as oxidative stress and heat shock."

1.6.2 General objectives

The general objectives of the project are to improve understanding of adaptive cellular stress responses. This thesis will evaluate whether induction of cellular defense systems at a mild non-lethal temperature can afford protection against activation of the apoptotic cascade induced by the pro-oxidant hydrogen peroxide.

1.6.3 Specific objectives

1) **Objective 1:** To determine whether mild thermotolerance (40°C, 3h) leads to induction of cellular defences such as antioxidants and anti-apoptosis proteins and protects cells against activation of the mitochondrial pathway of apoptosis by hydrogen peroxide in human cervical carcinoma cells (HeLa).

2) **Objective 2:** To evaluate the protective role of mild thermotolerance, developed at 40°C, against activation of the death receptor-mediated apoptotic cascade by pro-oxidant hydrogen peroxide in HeLa cells.

3) **Objective 3:** To investigate whether mild thermotolerance can alter the induction of ER-mediated stress and apoptosis in cells exposed to hydrogen peroxide.

CHAPTER II

EXPERIMENTAL RESULTS

(THREE ARTICLES)

2.1 PREFACE

This section includes three scientific articles describing the experimental results of this project which I have carried out during my doctorate studies in the laboratory of Dr. Diana Averill-Bates.

The first article entitled: **"Mild thermotolerance induced at 40°C increases antioxidants and protects HeLa cells against mitochondrial apoptosis induced by hydrogen peroxide: role of p53"** by Pallepati P and Averill-Bates DA. This article is published in the journal **Archives of Biochemistry and Biophysics 495 (2): 97-111, 2010.** Experiments were carried out by me and the manuscript was composed by myself and revised by Dr. Diana Averill-Bates.

The second article entitled: **"Mild thermotolerance induced at 40°C protects HeLa cells against activation of death receptor mediated apoptosis by hydrogen peroxide"** by Pallepati P and Averill-Bates DA. This article is published in the journal **Free Radical Biology and Medicine 50 (6): 667-79, 2011.** Experiments were carried out by me and the manuscript was composed by myself and revised by Dr. Diana Averill-Bates.

A third article entitled: **"Activation of ER stress and apoptosis by hydrogen peroxide in HeLa cells: protective role of mild heat preconditioning at 40°C"** by Pallepati P and Averill-Bates DA. This article is published in the journal **Biochimica Biophysica Acta – Molecular Cell Research 1813 (12): 1987-1999, 2011.** Experiments were

carried out by me and the manuscript was composed by myself and revised by Dr. Diana Averill-Bates.

Book Chapter: Pallepati P, Averill-Bates DA. **"Reactive oxygen species, cell death signaling and apoptosis".** Invited book chapter. **Principles of Free Radical Biomedicine. Kostas Pantopoulos and Hyman Schipper, eds., Nova Sciences Publishers, Inc., Hauppauga, New York, In press 2011.** The following contents of the book chapter, Overview of apoptosis and cell death, Signaling pathways of apoptosis, Role of calcium in ROS-mediated apoptosis, ROS and p53-mediated apoptosis, were written by myself and revised by Dr. Diana Averill-Bates. Some of these contents were included as a part of the Introduction section of this thesis.

Other Contributions: (i) **"Acrolein induces a cellular stress response and triggers mitochondrial apoptosis in A549 cells"** by Roy J, Pallepati P, Bettaieb A, Tanel A, Averill-Bates DA. My contribution in this article is that I helped Julie Roy (MSc) in repeating some of her experiments so that statistically significant data was obtained. I also carried out new experiments that were required by the evaluators in order to accept the manuscript. The manuscript was revised by Dr. Diana Averill-Bates. The article was published in the journal **Chemico-Biological Interactions 181 (2):154-67, 2009.** See Appendix A.

(ii) **"Acrolein induces apoptosis through the death receptor pathway in A549 lung cells: role of p53"** by Roy J, Pallepati P, Bettaieb A, Tanel A, Averill-Bates DA. My contribution in this article is that I helped Julie Roy (MSc) in repeating some of her experiments so that statistically significant data was obtained. I also carried out new experiments that were required by the evaluators in order to accept the manuscript. The manuscript was revised by Dr. Diana Averill-Bates. The article was published in the journal **Canadian Journal of Physiology and Pharmacology 88 (3):353-68, 2010.** See Appendix B

(iii) **"Acrolein causes ER stress and apoptosis in A549 lung cells"** by Andre Tanel, Pragathi Pallepati, Patrick Morin, Yulia Zilber and Diana A. Averill-Bates. My contribution in this article is that I wrote the material and methods and results sections whereas the Introduction and Discussion sections were written by Andre Tanel. Experiments were carried out by Yulia Zilber and I supervised experiments carried out by Patrick Morin. The manuscript is under revision by Dr. Diana Averill-Bates.

2.2 ARTICLE I

MILD THERMOTOLERANCE INDUCED AT 40°C INCREASES ANTIOXIDANTS AND PROTECTS HELA CELLS AGAINST MITOCHONDRIAL APOPTOSIS INDUCED BY HYDROGEN PEROXIDE: ROLE OF P53

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Short title: Themotolerance, antioxidants and apoptosis

Note: References are included in the general reference section.

RÉSUMÉ

L'exposition des cellules à des températures modérées (40°C) induit la tolérance thermique, ce qui rend les cellules résistantes ultérieurement aux stress toxiques. La tolérance thermique est habituellement associée à l'accumulation de protéines de choc thermique. Cette étude détermine si la tolérance thermique modérée (40°C, 3h) peut induire d'autres protéines de défense (par exemple des antioxydants, des protéines anti-apoptotiques) et protéger les cellules HeLa contre l'apoptose déclenche par le H_2O_2. L'expression des protéines MnSOD et catalase ainsi que leur activité enzymatique sont augmentées dans les cellules tolérantes à la chaleur. De plus, les niveaux intracellulaires de glutathion et l'expression de la γ-glutamylcystéine synthétase sont également augmentés. En outre, les niveaux d'espèces réactives d'oxygène (ROS) ont été augmentés dans les cellules tolérantes à la chaleur, ce qui cause l'hyperpolarisation de la membrane mitochondriale. La tolérance thermique modérée inhibe l'activation de la cascade apoptotique via la mitochondrie induite par le H_2O_2. Cette inhibition entraîne une translocation de Bax mitochondrial, une dépolarisation de la membrane mitochondriale, la sortie du cytochrome c, l'activation des caspases-9/-3 et la condensation de la chromatine. La tolérance thermique inhibe l'apoptose indépendante des caspases induite par le H_2O_2, impliquant le AIF, l'activation de p53 et l'expression accrue de sa protéine cible PUMA. La tolérance thermique induite aux températures physiologiques modérées protège les cellules tant contre l'apoptose dépendante des caspases que contre l'apoptose indépendante des caspases déclenche par le stress oxydatif.

Mot-clé: Stress oxydatif; Peroxyde d'hydrogène; Tolérance thermique; protéine de choc thermique; Apoptose; Caspases; Antioxydant; Mitochondrie; protéine p53

ABSTRACT

Exposure of cells to mild temperatures (40°C) induces thermotolerance, which renders cells resistant to subsequent toxic insults. Thermotolerance is usually associated with accumulation of heat shock proteins. This study determines whether mild thermotolerance (40°C, 3h) can induce other defense proteins (e.g. antioxidants, anti-apoptosis proteins), and protect HeLa cells against apoptosis triggered by H_2O_2. Protein expression and enzymatic activity of MnSOD and catalase were increased in thermotolerant cells, as well as intracellular glutathione levels and γ-glutamylcysteine synthetase expression. Furthermore, levels of reactive oxygen species (ROS) were increased in thermotolerant cells, which caused mitochondrial membrane hyperpolarisation. Mild thermotolerance inhibited activation of the mitochondrial cascade of apoptosis by H_2O_2. This entailed inhibition of mitochondrial Bax translocation, mitochondrial membrane depolarisation, cytochrome c release, activation of caspases-9/-3 and chromatin condensation. Thermotolerance inhibited H_2O_2-induced caspase-independent apoptosis involving apoptosis-inducing factor, and activation of p53 and increased expression of its target protein PUMA. Thermotolerance induced at mild physiological temperatures protects cells against both caspase-dependent and caspase-independent apoptosis triggered by oxidative stress.

Keywords: Oxidative stress; Hydrogen peroxide; Thermotolerance; Heat shock protein; Apoptosis; Caspases; Antioxidant; Mitochondria; p53 protein

INTRODUCTION

Reactive oxygen species (ROS) include partially reduced metabolites of oxygen such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). These species, whether generated as byproducts of mitochondrial respiration or as a consequence of exposure to environmental factors (e.g. ionising radiation, UV rays, heavy metals, cigarette smoke, pollutants, pesticides and anticancer drugs), pose a constant threat to cells and tissues living in an aerobic environment and can be toxic when present at higher doses [Thannickal et al, 2000]. Cells cope with the toxicity of ROS by using a range of non-enzymatic (e.g. glutathione, vitamin E, ascorbate, pyruvate, flavonoids, and carotenoids) and enzymatic antioxidants that include superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx). These antioxidants function by either scavenging ROS or by repairing ROS-induced damage. Superoxide radicals produced by the mitochondrial respiratory chain are dismutated by mitochondrial SOD (MnSOD) [Fridovich, 1997], leading to the production of H_2O_2, which is further detoxified to water, either by catalase or glutathione peroxidase (GPx) [Radi et al, 1991; Chance et al, 1979] These antioxidants counteract and regulate overall ROS levels to maintain physiological homeostasis. Oxidative stress occurs when this critical balance is disrupted, which can result from excess ROS production, depletion of antioxidants, or both. Oxidative stress has been implicated in the etiology of wide array of human diseases such as atherosclerosis, cancer, diabetes, arthritis, neurodegenerative disorders and aging [Finkel and Holbrook, 2000]. It is therefore important to clarify the underlying mechanisms in order to elucidate successful preventive and/or protective measures against ROS in disease processes as well as in environmental exposures.

H_2O_2 is considered to be a key ROS because of its involvement in cellular signaling cascades. Although the mode of action of H_2O_2 has been widely studied, the precise molecular mechanisms involved in apoptosis are not entirely characterized and the role of

H_2O_2 in cellular signalling remains unclear. At nontoxic submicromolar levels, H_2O_2 has been implicated in cellular functions such as growth, proliferation and differentiation [Burdon, 1995]. However, at higher micromolar to millimolar concentrations, depending on the cell type, H_2O_2 can cause oxidation, such as lipid peroxidation and protein oxidation, as well as depletion of levels of ATP, reduced glutathione and NADPH. Eventually, irreversible damage to cellular targets leads to cell death by processes such as apoptosis or necrosis [Chandra et al, 2000; Groeger et al, 2009]. In general, higher concentrations of hydrogen peroxide induce necrosis, whereas lower concentrations induce apoptosis [Troyano et al, 2003]. During apoptosis, cells become fragmented into apoptotic bodies that are recognized and engulfed by macrophages, thereby avoiding an inflammatory response in surrounding tissues, which clearly distinguishes it from necrotic cell death [Savill and Fadok, 2000]. During necrosis, due to swelling, disruption of organelles, and the loss of membrane integrity, the cellular contents are released uncontrolled into the extracellular environment, resulting in damage to surrounding cells and tissue, and a strong inflammatory response [Leist and Jaattela, 2001].

Hyperthermia (42-47°C) is among the recent and innovative methods of cancer treatment [Horsman and Overgaard, 2007]. Although temperatures exceeding 42°C are cytotoxic to cells, hyperthermia is mainly used as an adjuvant to radiation and chemotherapy. Localised heating allows targeting of radiation and chemotherapy treatments to the tumor region. Hyperthermia is one of the most effective radiation sensitizers known and can eliminate radio-resistant tumor cells. It can enhance the cytotoxic effects of certain anticancer drugs including bleomycin, Adriamycin, melphalan and platinum derivatives. However, the preconditioning of cells at elevated temperatures induces a stress response known as thermotolerance, which renders cells resistant to subsequent lethal insults such as heat shock, oxidative stress, chemotherapeutic agents, radiation and environmental stressors [Gill et al, 1998; Martindale and Holbrook, 2002; Kregel, 2002]. Thermotolerance can be developed following shorter exposures (e.g. 30

min) to lethal temperatures (42-45°C), or during continuous heating (e.g. 3-24h) at non-lethal temperatures (39.5-41.5°C) [Wiese et al, 1995; Landry et al, 1982].

Thermotolerance is transient and usually declines within several days, thus there is no interference with clinical use of hyperthermia. Most studies have investigated the induction of thermotolerance at higher lethal temperatures such as 42 to 45°C, whereas induction by lower temperatures has received little attention. Mild temperatures such as 40°C are physiological and occur during fevers. Thermotolerance is generally associated with the synthesis and accumulation of heat shock proteins (Hsp) [Landry et al, 1982; Przybytkowski et al, 1986; Landry et al, 1989].

Thermotolerance could also induce other cellular defenses such as antioxidants and anti-apoptosis proteins. Therefore, this study determines whether mild thermotolerance developed at 40°C can induce major antioxidants such as SOD, catalase and glutathione, and anti-apoptosis factors such as Bcl-2 family proteins and inhibitor of apoptosis proteins (IAPs). Furthermore, the ability of mild thermotolerance to protect cells against activation of apoptosis is not well understood. This study also evaluates whether thermotolerance developed at 40°C can afford protection against H_2O_2-induced activation of mitochondrial apoptosis in human cervical carcinoma (HeLa) cells. The role of p53 is also considered, along with the protective role of mild thermotolerance against both caspase and caspase-independent cell death processes mediated by H_2O_2.

MATERIALS AND METHODS

Cell culture

HeLa cells (ATCC#CCL-2) were grown in monolayer in Dulbecco's modified Eagle's medium (Invitrogen Canada, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen), in tissue culture flasks (Sarstedt, St Laurent, QC, Canada) at 37°C in a humidified atmosphere of 5% CO_2 in a water jacketed incubator [Bettaieb and Averill-Bates, 2008]. Medium was replaced with fresh medium 24h before experiments. To induce thermotolerance, cells were transferred to an identical incubator for 3h at 40°C (± 0.1°C), following a period of 20 min to allow the temperature of the culture medium to reach 40°C [Przybytkowski et al, 1986]. The cells were grown to near confluence and then harvested using 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution, and washed by centrifugation (1000 x g, 3 min). There was no loss of viability in cells heated at 40°C for 3h, evaluated by trypan blue exclusion (data not shown).

Treatment with H_2O_2 and inhibitors

Confluent cells in monolayer were pretreated or not with the following inhibitors: pifithrin-α (10 μM, 1h), a reversible inhibitor of p53-mediated apoptosis (Calbiochem, La Jolla, CA, USA), polyethylene glycol-catalase (PEG-catalase) (300 U/ml, 3h) (cell permeable H_2O_2 scavenger) (Sigma-Aldrich Canada) and L-buthionine sulfoximine (L-BSO) (1 mM, 3h (Sigma-Aldrich Canada)). Freshly harvested thermotolerant and non-thermotolerant HeLa cells were then resuspended in D-MEM plus 10% FBS and incubated at constant cell density ($1x10^6$/ml) with 25 to 100 μM of H_2O_2, in a final volume of 1.0 ml at 37°C for 1h or 3h, relative to untreated controls. Cells were washed by centrifugation (1000 x g, 3 min) to remove H_2O_2, and then analysed for mitochondrial apoptosis and clonogenic cell survival.

Flow cytometric analysis of mitochondrial membrane potential

The collapse of electrochemical gradient across the mitochondrial membrane is one of the early events during cellular apoptosis. The mitochondrial membrane potential was detected using 5,5',6,6'-tetrachloro-1,1',3,3'tetraethylbenzimidazolcarbocyanine (JC-1; Molecular Probes, Eugene, OR, USA). JC-1 forms red fluorescent aggregates in energized mitochondria with high membrane potential, whereas it dissociates to monomers with green fluorescence at low membrane potential. The aggregate red form has absorption/emission maxima of 585/590nm. The green monomeric form has absorption/emission maxima of 510/527 nm. Healthy, apoptotic, and necrotic cells can be analysed simultaneously by flow cytometry using different detectors. Following treatment with H_2O_2, thermotolerant (3h, 40°C) and non-thermotolerant (3h, 37°C) HeLa cells (1×10^6) were rinsed once with complete culture medium and incubated with 10 μM of JC-1 for 30 min at 37°C. Cells were washed twice and resuspended in 500 μl of cold PBS. Cells were analysed with a FACScan flow cytometer (Ex. 488 nm) equipped with an argon laser (Becton Dickinson, Oxford, UK) [Bettaieb and Averill-Bates, 2008]. Data was acquired and analysed using Lysis II software (Becton Dickinson). The mean fluorescence intensity of 10,000 cells was calculated for each sample and corrected for autofluorescence obtained from samples of unlabelled cells. The ratio of red fluorescence (detected by FL-2 detector) to green fluorescence (FL-1 detector) was considered as the relative $\Delta\Psi m$ value. The chemical uncoupler p-trifluoromethoxy-phenyl-hydrazone (FCCP, 25μM, 30 min) was used as a positive control for the $\Delta\Psi m$ decrease.

ROS generation

Cells were harvested after 3h at 37°C or 40°C, and then 10 μM 2′7′-dihydrodichloro-fluorescein diacetate (H_2DCFDA) or 20 μM dihydroethidium (DHE) was added for 30 min. H_2DCFDA measures steady state levels of pro-oxidants (presumably hydroperoxides), whereas DHE estimates intracellular superoxide levels. Levels of ROS

were determined in 10 000 cells by flow cytometry using the FL-1 detector and corrected for autofluorescence from unlabelled cells [Bettaieb and Averill-Bates, 2008].

Preparation of whole cell lysates

For analysis of total SOD activity, and protein expression of MnSOD, CuZnSOD, catalase, gamma-glutamyl cysteine synthetase (γ-GCSc), Bcl-2, c-Flip, C-IAP1/2, survivin, Bax, Bid, Bad and Bip, cells were harvested after 3h at 37°C or 40°C. For detection of p53, PUMA, ICAD, and PARP proteins, thermotolerant and non-thermotolerant cells were harvested and incubated with hydrogen peroxide at 37°C for the required time periods. Cells were washed by centrifugation (1000 x g, 3min) in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) [21]. The supernatant was discarded, pelleted cells were resuspended in lysis buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 1h. Then, by a 10 min centrifugation step at 2500 x g to remove nuclei and unbroken cells, the proteins of whole cell lysates were isolated in the supernatant [Bettaieb and Averill-Bates, 2008] for immunodetection of proteins and detection of total SOD activity.

Preparation of subcellular fractions

Cells were pretreated with or without 10 μM pifithrin-α for 3 h and then exposed to H_2O_2. Subcellular fractions were then prepared as previously described [Bettaieb and Averill-Bates, 2008; Samali et al, 1999]. Thermotolerant and non-thermotolerant cells were washed in buffer A and resuspended in buffer B containing 0.1 mM dithiothreitol (DTT). Lysates were homogenised using a dounce homogeniser (50 strokes/sample). After a 30 min incubation on ice, unbroken cells and nuclei were pelleted by centifugation at 2500 x g for 10 min. The nuclear fraction was purified and isolated from the cellular debris, and was used for the detection of apoptosis inducing factor (AIF). The

supernantant was centrifuged further at 15,000 x g for 15 min.The pellet containing the mitochondrial fraction was then resuspended in buffer C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS, 0.1 mM DTT, 100 µl/10 ml of cocktail of protease inhibitors, pH 7.4) and used for detection of Bax and AIF. The supernatant was further centrifuged at 100 000 x g for 1h. The resulting supernatant was designated as the cytosolic fraction which was used for detection of Bax, cytochrome c, and AIF. Purity of nuclear (89%), cytosolic (98%) and mitochondrial (97%) fractions was determined by Western blotting using lamin B, glutathione S-transferase (GST-π1) and cytochrome oxidase, respectively.

Immunodetection

Proteins (40 µg) were quantified according to Bradford [Bradford, 1976] and then solubilised in Laemmli sample buffer [Laemmli, 1970]. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8% for PARP; 10% for AIF, catalase, γ-GCSc and Bip; 15% for CuZnSOD, MnSOD, Gpx, Bid, Bcl-2, C-Flip, Survivin, Bax, Cytochrome c, p53 and PUMA) of cellular proteins was carried out at a constant voltage of 125 V. Cellular proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Milli-pore, Bedford, MA, USA) [Tanel and Averill-Bates, 2007]. The blots were probed with the following primary antibodies (1:1000): anti-PARP, anti-AIF, anti-catalase, anti-γ-GCSc, anti-Gpx, anti-Bcl-2, anti-C-Flip, anti-Survivin, anti-Bax, anti-Bad, anti-cytochrome c, anti-PUMA, anti-GAPDH (1: 5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-CuZnSOD, anti-MnSOD, and anti-p53 $Ser^{15,46}P$ (Stressgen, San Diego, CA, USA), anti-Bip (BD Biosciences Canada, Mississauga, ON, Canada) in Tris-buffered saline (50 mM Tris base, 150 mM NaCl, 0.1% Tween-20) (TBS-T) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed and incubated for 1h at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-mouse, or anti-rabbit, IgG (1:1000) (Biosource, Camarillo, CA, USA) diluted in TBS-T containing 5% milk powder. Proteins were detected using the ECL plus chemiluminescence kit

(PerkinElmer, Boston, MA, USA). Protein expression was analysed using a scanning laser densitometer (Alpha Innotech Corp., San Leandro, CA, USA) and Fluorchem software, relative to GAPDH.

Caspase activity by fluorescence spectroscopy

Following treatment with H_2O_2 (0, 25-100 μM) for 3h, freshly harvested thermotolerant and non-thermotolerant HeLa cells were washed twice with cold PBS by centrifugation (1000 x g, 3 min at 4°C) and resuspended in 75 μl of reaction buffer (20 mM piperazine-N,N′-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane-sulfonic acid (CHAPS), 10% sucrose, pH 7.2) [Tanel and Averill-Bates, 2007]. Cells were lysed at –80°C for 30 min. The kinetic reaction was followed for 30 min after addition of the appropriate caspase substrate at a final concentration of 50 μM, at 37°C using a Spectra Max Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, CA). Caspase-9 activity was measured by cleavage of the fluorogenic peptide substrate Ac-Leu-Glu-His-Asp-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC (Calbiochem, La Jolla, CA, USA) to produce 7-amino-4-trifluoromethylcoumarin (AFC) with λmax excitation at 400nm and λmax emission at 505nm. For caspase-3, the substrate was Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) with λmax excitation at 380 nm and λmax emission at 460nm. Activities of caspases are represented as Vmax of the kinetic reaction.

Catalase activity

Freshly harvested thermotolerant and non-thermotolerant HeLa cells (5 x 10^6), were lysed via sonication (twice for 20 s) (Vibra Cell, Sonics & Materials, Inc., Danbury, CT) in 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (14,000 rpm, 15 min) to obtain a clear extract [Lord-Fontaine and Averill-Bates, 1999; Claiborne, 1985]. The dosage was performed using a solution of 19 mM H_2O_2 containing the clear cellular

extract in a final volume of 3 ml of phopshate buffer. Catalase activity in the cellular extract was measured as hydrogen peroxide consumption/min [Claiborne, 1985], by detecting the decrease in absorbance at 240 nm during 120 s and recording over 15-s intervals, using a DU-62 spectrophotometer (Beckman). One unit was defined as μmol of peroxide consumed/min/10^6 cells, obtained using the intial velocity from the linear slope of the curve. Catalase activity was normalized to controls and displayed as fold difference.

Glutathione peroxidase enzymatic activity

Glutathione peroxidase activity was measured by following the oxidation of NADPH spectrophotometrically at 340nm [Lord-Fontaine and Averill-Bates, 1999; Lawrence and Burk, 1976]. Cells were resuspended in 500 μL of PBS, sonicated twice for 20 sec and then centrifuged (10,000 x g, 15 min). Cell extracts were added to 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 mM GSH and glutathione reductase (1.3 E.U./mL). The mixture was incubated for 10 min at 25°C before the addition of 0.25 mM H_2O_2. The kinetic reaction was followed at 340 nm at 15-s intervals for 5 min. One unit was defined as μmol of peroxide consumed/min/10^6 cells, obtained using the intial velocity from the linear slope of the curve. GPx activity was normalized to controls and displayed as fold difference.

Enzymatic activity of SOD by zymography

Whole cell lysates were prepared (see 2.5 above) and used to measure total SOD activity in thermotolerant and non-thermotolerant HeLa cells. Total SOD activity (absence of cyanide) was investigated by zymography as described by Beauchamp and Fridovich [Beauchamp and Fridovich, 1971], using riboflavin as a reducing agent. Equal amounts of protein (30 μg) were electrophoresed on 12% non-denaturing polyacrylamide gels. After electrophoresis, gels were stained in the dark for 15 min with nitroblue tetrazolium (NBT), in the presence of TEMED and riboflavin. The gels were then

exposed to light for approximately 30 min. SOD activity was observed as discoloured bands on a purple colored background. Densitometric analyses were performed and band densities were compared estimating the relative enzymatic activities.

Measurement of total glutathione

Freshly harvested thermotolerant (40°C, 3h) and non-thermotolerant (37°C, 3h) HeLa cells (1 x 10^6) were used for the detection of glutathione. Total glutathione was determined according to the user's manual for the ApoGSHTM Glutathione Colorimetric Detection Kit (BioVision). Briefly, cells (5 x 10^5 cells) were centrifuged at 700 x g for 5 min at 4°C and the supernatants removed. The pellets were washed with ice-cold PBS, lysed in 80 µl of ice-cold Glutathione Buffer and incubated on ice for 10 min. Then the samples were dissolved with 5% 5-sulfosalicylic acid (SSA, 20 µl) and centrifuged at 8000 x g for 10 min. The supernatants (20 µl) were incubated in 160 µl of the Reaction Mix at room temperature for 10 min and Substrate solution (20 µl) was added and the mixture was incubated for a further 10 min. Absorbance was measured at 415nm using a micro plate reader. The standard curve was obtained from absorbance of the diluted GSH Standard that was incubated in the mixture as in samples.

Hoechst 33258 staining

To visualize nuclear morphology and chromatin condensation by fluorescence following treatment with H_2O_2, freshly harvested thermotolerant and non-thermotolerant cells (1x10^6/ml) were washed twice with PBS and Hoechst 33258 (1 mg/ml) (blue-green fluorescence) (Sigma Chemical Co.), which binds to condensed chromatin in the nucleus of apoptotic cells, was added for 15 min at 37°C [Tanel and Averill-Bates, 2007]. Cells were washed with PBS and propidium iodide (red fluorescence) (Sigma Chemical Co.) (1mg/ml) was added subsequently to stain necrotic cells. Observations were made by fluorescence microscopy (Carl Zeiss Canada Ltd., St. Laurent, QC, Canada) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix

imaging Inc, Mississauga, ON). Images were analyzed using Northern Eclipse software. Cells were classified using the following criteria: (1) Live cells with normal nuclei, pale blue/green chromatin with organized structure); (2) Apoptotic cells with bright blue /green condensed or fragmented chromatin) (3) Necrotic cells (red, enlarged nuclei with smooth normal structure [Tanel and Averill-Bates, 2007]. The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright field illumination). A minimum of 300 cells was counted per sample.

Clonogenic cell survival assay

Clonogenic cell survival measures the ability of cells to undergo cell proliferation following a toxic insult. Non-thermotolerant cells, with and without pifithrin-α (10μM, 3h), and thermotolerant (40°C, 3h) cells were exposed to H_2O_2 (0-100 μM) for 2h at 37°C in a final volume of 1.0 ml, in D-MEM containing 10% FBS. After the appropriate time, the cells were washed three times by centrifugation (1000 x g, 2 min) to stop the incubation [Lord-Fontaine and Averill-Bates, 1999]. The cells were resuspended in culture medium, diluted to the appropriate concentration and plated in tissue culture dishes (60 mm x 15 mm), which were incubated at 37°C incubator in an atmosphere of 5% CO_2 for 10 days. The dishes were then washed with PBS, fixed with 95% ethanol and stained with methylene blue before counting macroscopic colonies (>50 cells). Cytotoxicity was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells were seeded in the control plates, but where there was a loss of cell survival, cells were plated at several different densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have previously demonstrated that, in this system, there is linearity between the number of cells plated and colonies formed over the range of $10\text{-}10^4$ [Lord-Fontaine and Averill-Bates, 1999].

Statistics

Data represent means ± SEM from at least 3 independent experiments. When not shown, error bars lie within symbols. Comparisons of mean values with the control were analysed by the Student's bilateral *t* test. The Bonferroni-Holmes stepwise adjustment was used to control for the Family-wise error rate at a desired level (α=5%). Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means, with Dunnett adjustment. Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). For significant differences, $p<0.05$.

RESULTS

Mild thermotolerance developed at 40°C increases the expression of major antioxidants

The development of thermotolerance at a mild temperature of 40°C for 3h was shown to induce the protein expression of six major Hsps of 27, 32, 60, 72, 90 and 110 kDa [Bettaieb and Averill-Bates, 2008]. The present study determines whether exposure of cells to a fever temperature of 40°C for 3h could induce the expression of other cellular defense proteins, such as antioxidants or anti-apoptosis proteins. Antioxidants such as SOD, catalase, GPx and glutathione play a major role in ROS regulation and hence, their levels were compared between thermotolerant and non-thermotolerant HeLa cells (Figs. 1-3). The enzymatic activities of SOD (Fig. 1A and 1B) and catalase (Fig. 2A) in whole cell lysates were increased significantly by 38 and 49%, respectively, in thermotolerant cells compared to control cells. Mild thermotolerance also significantly increased the protein expression of MnSOD (41%) (Fig. 1C and 1D) and catalase (46%) (Figure 2B and 2C). At 40°C, there was a small increase in CuZnSOD expression (5%) (Fig. 1E and 1F). Furthermore, intracellular glutathione levels were three times higher in thermotolerant cells, compared to non-thermotolerant cells (Fig. 3A). Since γ-glutamyl cysteine synthetase (γGCS) is the rate-limiting enzyme in glutathione synthesis, we

determined whether mild thermotolerance could alter its expression. Indeed, the protein expression of γ-GCS increased by 40% in thermotolerant cells compared to controls (Fig. 3B and 3C). However, there was no change in the activity and expression of GPx, the other peroxide detoxifying antioxidant (Fig. 3D-3F). These results show that mild thermotolerance induces not only the expression of Hsps, but also the expression and enzymatic activity of several major antioxidants.

Subsequently, the expression of anti-apoptosis proteins was verified in thermotolerant cells. However, mild thermotolerance (3h, 40°C) did not alter the expression of anti-apoptotic proteins such as Bcl-2, Bip, c-FLIP, cIAP1/2 and survivin, or pro-apoptotic proteins such as Bax, Bid and Bad, from control levels (data not shown).

Cellular levels of ROS are increased during mild thermotolerance (40°C)

The induction of antioxidants during thermotolerance suggests that exposure to mild heat shock at 40°C for 3h could increase ROS generation. Consequently, levels of ROS were compared in thermotolerant and non-thermotolerant cells (Fig. 4). Indeed, there was a 53% increase in H_2DCFDA fluorescence in thermotolerant cells, relative to controls at 37°C (Fig. 4A and 4B). Pretreatment of cells with PEG-catalase (300 U/ml, 3h) inhibited the increase in H_2DCFDA fluorescence, which suggests that mild heating at 40°C increased levels of H_2O_2. PEG-catalase increased intracellular catalase activity by 47% (Fig. 4C). However, there was no difference in levels of DHE fluorescence between thermotolerant and normal cells (Fig. 4D).

Mild thermotolerance developed at 40°C protects HeLa cells against H_2O_2-induced mitochondrial changes

Subsequently, the ability of mild thermotolerance (40°C) to protect cells against oxidative stress-induced apoptosis was investigated. ROS and a number of stimuli, including chemotherapeutic agents, UV radiation and growth factor withdrawal, can

mediate apoptosis via the «stress-activated» mitochondrial pathway [Chandra et al, 2000]. An early event in this pathway is the translocation of pro-apoptotic protein Bax from the cytosol to mitochondria. This leads to Bax oligomerisation and membrane insertion, which alters mitochondrial membrane potential by forming Bax pores [Lalier et al, 2007]. Indeed, exposure of HeLa cells to H_2O_2 (25-100 μM) for 1h caused a significant dose-dependent increase (30-80%) in Bax levels at mitochondria, relative to untreated control cells (Fig. 5A,5B). There was a corresponding decrease in cytosolic levels of Bax (data not shown). However, when thermotolerant (40°C) cells were treated with H_2O_2, Bax translocation to mitochondria was diminished significantly (Fig. 5A and 5B). This suggests that mild thermotolerance developed at 40°C inhibited H_2O_2-induced Bax translocation to mitochondria. The purity of mitochondrial and cytosolic fractions was 97 and 98%, respectively (Fig. 5C).

To determine whether Bax translocation is associated with disruption of mitochondrial transmembrane potential ($\triangle\Psi m$), HeLa cells were loaded with the cationic fluorochrome JC-1. The $\triangle\Psi m$ decreased as a function of dose in cells treated with H_2O_2 (25-100 μM) for 1h (Fig. 5D). FCCP was used as a positive control and caused a decrease in $\triangle\Psi m$ by 80%. $\triangle\Psi m$ was higher in thermotolerant cells compared to normal cells (Fig. 5D), and this appeared to represent a protective effect against the decrease in $\triangle\Psi m$ induced by H_2O_2 in non-thermotolerant cells. Membrane hyperpolarization occurred in untreated thermotolerant control cells, but there was a dose dependent decrease in $\triangle\Psi m$ when these cells were exposed to H_2O_2 (Fig. 5D). Several studies have linked mitochondrial membrane hyperpolarisation with ROS generation [Russell et al, 2002; Park et al, 2005; Roy et al, 2009]. Indeed, the increase in mitochondrial membrane potential observed in untreated thermotolerant cells was inhibited by PEG-catalase (Fig. 5E), suggesting that the increased ROS production detected in thermotolerant cells (Fig. 4A and 4B) could be responsible for this effect.

Once the mitochondrial membrane potential is altered, proteins located within mitochondria, such as cytochrome c and AIF, can gain access to the cytosol [Chandra et al, 2000]. When HeLa cells were treated with H_2O_2 (25-100 μM) for 2h, there was a significant dose-dependent increase in cytosolic levels of cytochrome c, relative to unteated controls (Fig. 6A and 6B). Mild thermotolerance developed at 40°C inhibited H_2O_2-induced cytochrome c release from mitochondria (Fig. 6A and 6B).

Once released into the cytosol, cytochrome c interacts with dATP, apoptosis protease activating factor (Apaf1) and pro-caspase-9, forming the apoptosome complex. Within the apoptosome, pro-caspase-9 is converted to active caspase-9, which subsequently causes proteolytic activation of effector caspases such as caspase-3 [Chandra et al, 2000]. Exposure of HeLa cells to H_2O_2 (25-100 μM) for 3h caused significant dose-dependent activation of initiator caspase-9 (Fig. 7A) and caspase-3, relative to untreated controls (Fig. 7B). Activity of these caspases decreased significantly in thermotolerant cells, compared to non-thermotolerant cells (Fig. 7A and 7B). These results show that preconditioning of cells with mild heat shock attenuates H_2O_2-induced caspase activation.

Mild thermotolerance (40°C) protects HeLa cells against pro-oxidant-induced activation of the execution phase of apoptosis

Caspase-3, once activated, plays a central role in execution of the apoptotic program and can cleave certain cytoplasmic, cytoskeletal and nuclear protein substrates such as lamins, fodrin and polyADP-ribose polymerase (PARP). PARP is a DNA repair enzyme that catalyzes the poly (ADP-ribosyl) ation of a variety of nuclear proteins with NAD as substrate, and is activated by DNA strand breaks [Germain et al, 1999]. Cleavage of PARP by effector caspases inactivates the enzyme, which destroys its ability to respond to DNA strand breaks. The cell then exhibits the characteristic hallmark features of the execution phase of apoptosis such as chromatin condensation and DNA fragmentation. Subsequently, we determined if mild thermotolerance could inhibit activation of the

execution phase of apoptosis by H_2O_2. Protein levels of the 85kDa PARP cleavage fragment increased significantly in HeLa cells during exposure to H_2O_2 for 3h (Fig. 8A and 8B). Nevertheless, PARP cleavage was diminished significantly in thermotolerant cells.

Nuclear changes of the execution phase were detected using the fluorescent dye Hoechst 33258 (blue-green), which stains condensed chromatin in the nucleus of apoptotic cells. PI (red fluorescence) was used as a counter stain for necrosis. H_2O_2 (25-100 μM, 3h) induced apoptosis (11-34%) in a dose-dependent manner (Fig. 9A2-9A5, 9C), relative to control cells (Fig. 9A1). It should be noted that at higher peroxide concentrations (75 and 100 μM), there was an increase in necrosis (Fig. 9A4, 9A5, 9D), indicating a gradual switch in the mode of cell death from apoptosis to necrosis. Levels of apoptosis and necrosis were reduced in thermotolerant cells (Fig. 9B1-9B5, 9C, 9D), indicating that mild thermotolerance (40°C) protects against induction of apoptotic and necrotic cell death by H_2O_2. The induction of apoptosis by H_2O_2 was significantly attenuated by pretreatment of cells with 300 U/ml of PEG-catalase (Fig. 9E and 9F), a ROS scavenger that detoxifies H_2O_2.

Inhibition of glutathione synthesis during thermotolerance partially decreases development of resistance to H_2O_2-induced PARP cleavage

Together, the above results show that mild thermotolerance (40°C) protects cells against induction of mitochondria-mediated apoptosis by H_2O_2. To confirm the role of antioxidant induction by mild heat at 40°C in the development of resistance to H_2O_2-induced apoptosis, cells were treated with L-BSO, an inhibitor of γ-GCS. L-BSO was present during the 3h heating period at 40°C, to prevent any new synthesis of glutathione during the development of thermotolerance. PARP cleavage mediated by H_2O_2-treatment was evaluated, being a late stage event of apoptosis. Indeed, the inhibition of H_2O_2-induced PARP cleavage in thermotolerant cells was partially reversed when cells were

treated with L-BSO (Figure 10). This was seen as an increase in expression of the PARP cleavage fragment (85 kDa) in L-BSO-treated thermotolerant cells, compared to thermotolerant cells alone. In contrast, treatment of non-thermotolerant cells with L-BSO for 3h at 37°C did not increase H_2O_2-induced PARP cleavage (Figure 10). In fact, PARP cleavage by the higher dose of H_2O_2 75 (μM) was decreased with L-BSO. L-BSO did not affect levels of PARP fragment in untreated (no H_2O_2) controls during 3h at 37 or 40°C. These data indicate that the antioxidant glutathione contributes partially to the protective effect of mild thermotolerance against H_2O_2-induced apoptosis. However, we cannot rule out the contribution of other antioxidants such as MnSOD and catalase in this protective effect.

H_2O_2 activates caspase-independent apoptosis involving AIF: protective effect of mild thermotolerance induced at 40°C

Apoptosis can proceed through pathways that involve the cysteinyl aspartate-specific proteases (caspases), or through caspase-independent pathways [Leist and Jaattela; 2001]. AIF, located within the mitochondrial inner membrane, plays a key role in caspase-independent cell death processes, when released from mitochondria and translocated to the nucleus [Modjtahedi et al, 2006; Cregan et al, 2004]. AIF can bind directly to DNA, which stimulates its DNAse activity, leading to chromatin condensation and large-scale DNA fragmentation. Thus, mitochondrial proteins, once released, can trigger at least two different death-signaling pathways, by caspase-dependent and caspase-independent mechanisms. To determine the protective role of mild thermotolerance against this caspase-independent pathway in H_2O_2-treated HeLa cells, the translocation of AIF from mitochondria to the cytosol and nucleus was analyzed by Western blot. Effectively, exposure to H_2O_2 (25-100 μM) for 1h caused significant dose-dependent translocation of AIF to the nucleus (Fig. 11C and 11D) via the cytosol (Fig. 11A and 11B). In thermotolerant cells, AIF translocation to the nucleus was decreased significantly compared to their non-thermotolerant counterparts (Fig. 11A-11D). These findings

indicate that mild thermotolerance affords protection against caspase-independent cell death triggered by ROS.

Role of p53 in H_2O_2-induced apoptosis

The role of p53 as a transcription factor has been extensively described. It plays an important role in a wide variety of cellular functions like DNA repair, cell cycle arrest and apoptosis [Schuler and Green, 2001]. p53 is activated by different stress conditions, which include ROS, DNA damage, nucleotide depletion, hypoxia and oncogene activation. Post-translational modifications such as phosphorylation and acetylation play a key role in its pro-apoptotic activity. As a transcription factor, phosphorylated p53 induces transcription of pro-apoptotic proteins such as Bax and the BH3-only proteins Noxa and PUMA, and it suppresses the activity of anti-apoptotic proteins such as Bcl-2 [Miyashita and Reed, 1995; Yu et al, 2003]. We therefore determined whether induction of apoptosis by H_2O_2 in HeLa cells could be mediated by p53. The activation of p53 was assessed by looking at its phosphorylation status using phospho-specific antibodies. Exposure of cells to H_2O_2 (50 μM) for 1h caused significant p53 phosphorylation at Ser46 and Ser15 (Fig.12A-12C). Mild thermotolerance (40°C, 3h) exerted a protective effect by decreasing the level of p53 phosphorylation (Fig. 12A-12C). There was no change in the expression of p53 protein in non-thermotolerant and thermotolerant HeLa cells, with or without peroxide treatment (Fig. 12A and 12D). In order to clarify further the role of p53 in H_2O_2-induced apoptosis in HeLa cells, we studied the downstream targets of p53 such as PUMA and Bax. Indeed, H_2O_2 induced upregulation of the p53 target protein PUMA (Fig. 13A-13B), as well as Bax translocation to mitochondria (Fig. 5A and 5B, 13C and 13D). The role of p53 was confirmed by significant inhibition of these events by a p53 inhibitor, pifithrin-α (Fig. 13A-13D). Furthermore, PUMA upregulation and Bax translocation were inhibited in thermotolerant cells (Fig. 13A-13D). Treatment with pifithrin-α also diminished significantly H_2O_2-induced chromatin condensation (Fig. 14A and 14B). These results show that p53 is an upstream factor

leading to PUMA upregulation and mitochondrial Bax translocation, which favor mitochondria-mediated apoptosis in H_2O_2-treated HeLa cells.

Protective effect of mild thermotolerance against H_2O_2-induced cytotoxicity

We next determined whether mild thermotolerance could protect cells against clonogenic cell killing (cytotoxicity) under conditions where mild hyperthermia afforded resistance to H_2O_2-induced apoptosis. Exposure to H_2O_2 (0-150μM) caused a marked decline in clonogenic cell survival, and in thermotolerant cells, there was a significant protective effect (p=0.0002) (Fig. 14C). Subsequently, we evaluated whether inhibition of p53 with pifithrin-α could protect against H_2O_2-induced clonogenic cell killing. There was a tendency for a small protective effect against cytotoxicity induced by higher concentrations of H_2O_2 (100-150 μM) in cells that were treated with p53 inhibitor pifithrin-α (Fig. 14C).

DISCUSSION

Mild thermotolerance induced at 40°C increases ROS generation and expression of major antioxidants in HeLa cells

The present study shows that protein expression and enzymatic activity of two major antioxidants, MnSOD and catalase, were increased in HeLa cells following the development of thermotolerance at a non-lethal, fever temperature of 40°C for 3h. Intracellular levels of glutathione were also increased in thermotolerant cells and this could be explained by increased expression of γGCSc. The exposure of rat myocardium to whole body hyperthermia (42°C, 15 min) led to a delayed increase in MnSOD content, which was revealed 24 to 72h after the heat treatment [Yamashita et al, 1998].

The induction of antioxidants, such as catalase, MnSOD and glutathione, during the development of thermotolerance suggests that exposure to mild heat shock at 40°C for 3h

could increase ROS generation. Indeed, ROS levels (H_2DCFDA fluorescence) were increased at 40°C in HeLa cells. An interesting point is that the 45% increase in catalase activity observed after exposure to 40°C for 3h was not enough to fully prevent the increased peroxide formation (H_2DCFDA fluorescence), while an additional 47% of catalase activity achieved by incubation with PEG-catalase was effective. However, the increase in catalase activity is probably a consequence of increased H_2O_2 formation during the heat treatment at 40°C. This situation could be also explained in terms of intracellular compartments. Catalase is localised in peroxisomes, but it is not clear where PEG-catalase would be localised once inside the cell. This could be in the cytosol, or other compartments, since PEG allows catalase to cross biological membranes. In addition, the intracellular site(s) of increased generation of ROS by heat is unknown, although mitochondria would be a likely source.

The increased ROS levels at 40°C in HeLa cells in turn led to mitochondrial membrane hyperpolarisation. The mechanisms involved in membrane hyperpolarisation are currently unknown. Several studies reported that exposure to heat shock at higher temperatures, such as 42 to 45°C for 20 to 60 min, led to increased free radical electron paramagnetic resonance (EPR) signals [Flanagan et al, 1998] and oxidative stress [Bettaieb and Averill-Bates, 2008; Lord-Fontaine and Averill-Bates, 1999; Li and Oberley, 1997; Lord-Fontaine and Averill-Bates, 2002; Spitz and Li, 1990; Shin et al, 2008]. Hyperthermia is able to activate redox-sensitive signaling and transcription factors, where the initial cellular response is primarily mediated by heat shock factors (HSF) [Gius et al, 2004]. Oxidative stress and heat exposure can induce common alterations in early response genes such as p53, p21, AP-1, NF-κB, etc., which suggests that heat shock could have similarities to other environmental stresses that induce oxidative stress [Gius et al, 2004]. Furthermore, there is considerable overlap in signalling pathways, such as Ras and mitogen activated protein kinases (MAPK), which are induced by oxidative stress and heat shock.

Low dose exposure to different stresses can lead to adaptive responses that allow cells and organisms to continue normal function in the face of an adverse stimulus [Holsapple and Wallace, 2008]. Adaptive responses often involve changes in gene and protein expression, including the induction of cellular defenses to enable the cell to survive [Davies, 2000]. Organisms have developed an intricate system of defense molecules (e.g. Hsps, antioxidants, IAPs, etc.) to protect themselves against diverse toxic and environmental stresses. If the adaptive response cannot protect the cell against an adverse stress exposure, then the cell will be eliminated by death processes such as apoptosis [Davies, 2000]. It is well established that thermotolerance developed at both lethal (>42°C) [Landry et al, 1982; Landry et al, 1989] and non-lethal (39.5-41.5°C) temperatures [Przybytkowski et al, 1986; Bettaieb and Averill-Bates, 2008; Field and Anderson, 1982] is associated with increased expression of Hsps. A novel finding in this study is that, in addition to Hsps, preconditioning of HeLa cells with mild heat shock (40°C) can also induce several major antioxidants.

Mild thermotolerance developed at 40°C protects HeLa cells against H_2O_2-induced mitochondrial apoptosis

Subsequently, our results show that mild thermotolerance (40°C) inhibited the molecular events involved in the mitochondrial cascade of apoptosis induced by pro-oxidant H_2O_2 in HeLa cells. This entailed inhibition of Bax translocation to mitochondria, loss of mitochondrial membrane potential, cytochrome c release, activation of caspase-9 and -3, PARP cleavage and nuclear chromatin condensation. The induction of mitochondrial apoptosis by H_2O_2 has been reported in several cellular models [Chandra et al, 2000; Barbouti et al, 2002; Antunes and Cadenas, 2001], although the signalling mechanisms involved are not completely understood. Furthermore, thermotolerant cells were able to maintain reproductive integrity following an H_2O_2 insult. These results are potentially significant to the ability of cancer cells to exhibit heat-induced cross

resistance to therapeutic agents, such as radiation and oxidant-generating drugs, which kill cancer cells via oxidative stress.

The protective effects of mild thermotolerance against peroxide-induced apoptosis and necrosis in HeLa cells could be attributed to both Hsps and antioxidants (Scheme 1). Hsps seem to have a complex role in regulation of apoptosis [Beere, 2004]. Hsp70 appears to inhibit apoptosis by interfering with events upstream of mitochondrial membrane permeabilization that ultimately decrease Bax activation and block cytochrome c release [Steel et al, 2004; Stankiewicz et al, 2005]. Hsp70 can also protect against oxidative injury [Musch et al, 1996; Chen et al, 1999a; Komatsuda et al, 1999]. Hsp27 can block mitochondrial ROS generation, thus suppressing the loss of membrane potential and cytochrome c release [Samali et al, 2001]. In addition, Hsp27 can upregulate glutathione levels, which helps in maintaining the cellular redox equilibrium [Mehlen et al, 1996]. Hsp27, Hsp70 and Hsp90 can inhibit apoptosome formation, thus preventing caspase-9 activation [Beere, 2004]. Hsp72 inhibited procaspase-3 processing and PARP cleavage induced by heat-shock, thus preventing the executionary events of the apoptotic process [Buzzard et al, 1998].

The mitochondrial antioxidant defense systems appear to play a critical role in the regulation of apoptosis [Ott et al, 2007]. The intracellular sites of ROS generation during mild heat stress are not presently known. Mitochondria are a likely candidate given that the respiratory chain is a major cellular source of ROS, mainly at Complexes I and III [Ott et al, 2007]. Increased content of cellular defense proteins such as MnSOD could thus play a protective role against apoptosis in H_2O_2-treated thermotolerant cells by buffering mitochondrial ROS production. Upregulation of MnSOD protected adenocarcinoma and neuronal cells against apoptosis induced by stresses such as UV, doxorubicin and tumor necrosis factor-α [Delhalle et al, 2002; Sompol et al, 2006]. Furthermore, overexpression of MnSOD attenuated cytochrome c release, caspase-9

activation and apoptotic cell death in mice after focal cerebral ischemia [Noshita et al, 2001]. The overexpression of the antioxidant enzymes MnSOD, CuZnSOD and Gpx protected cells against ionizing radiation injury [Sun et al, 1998]. The influence of catalase activity on apoptosis is less clear and both anti-apoptotic and pro-apoptotic roles have been found [Kahl et al, 2004]. The GSH redox status, particularly in mitochondria, appears to be a central player in apoptosis [Circu, 2008]. Opening of the mitochondrial transition pore (MTP) is redox-dependent. Depletion of GSH leads to MTP opening and mitochondrial apoptosis. The antioxidant N-acetylcysteine, which increases intracellular GSH levels, protected CHO cells against acrolein-induced mitochondrial apoptosis [Tanel and Averill-Bates, 2007]. Therefore, increased levels of GSH during thermotolerance could protect cells against the loss of mitochondrial membrane potential during peroxide-induced apoptosis. Our findings suggest that upregulation of antioxidants by mild temperatures such as 40°C could scavenge mitochondrial ROS and thus play a role in regulation of apoptosis induced by pro-oxidant stresses (Scheme 1).

H_2O_2 activates caspase-independent apoptosis involving AIF: protective effect of mild thermotolerance induced at 40°C

This study shows that H_2O_2 can induce both caspase-dependent and caspase-independent pathways of apoptosis in HeLa cells. Interestingly, AIF release into the cytosol and its translocation to the nucleus occurred rapidly after a 1h exposure to H_2O_2, whereas caspase activation occurred later, after 3h. The cytosolic and nuclear levels of AIF were considerably lower in thermotolerant cells, compared to their non-thermotolerant counterparts, indicating that mild temperatures could also protect against caspase-independent cell death mediated by H_2O_2. Hsp70, which is induced by mild thermotolerance, can neutralize AIF, either by inhibiting its release from mitochondria and/or sequestering it once leaked into the cytosol [Ravagnan et al, 2001; Ruchalski et al, 2003]. Apart from its pro-apoptotic role, recent studies show that AIF plays an anti-apoptotic role by maintaining integrity of components (Complex I) of the mitochondrial

electron transport chain and by regulating mitochondrial ROS production via its putative oxidoreductase and peroxide scavenging activities [Modjtahedi et al, 2006; Cregan et al, 2004]. Thus, low levels of AIF in cytosolic and nuclear fractions indicate that AIF could play a cytoprotective role by ensuring proper mitochondrial function in thermotolerant cells. Interestingly, continuous rather than bolus addition of H_2O_2 caused nuclear AIF translocation and caspase-independent apoptosis in Jurkat cells, under conditions where caspase-dependent apoptosis was inhibited through an iron-dependent mechanism [Barbouti et al, 2007]. AIF appears to be a safeguard death mechanism under conditions where there is ineffective activation of caspases [Cregan et al, 2004, Ravagnan et al, 2001].

Protective effect of mild thermotolerance against H_2O_2-induced p53 activation

Finally, our findings using pifithrin-α indicate that p53 is an upstream factor leading to increased expression of PUMA and Bax translocation to mitochondrial, during H_2O_2-induced apoptosis in HeLa cells. p53, as a transcription factor, is able to induce transcription of PUMA, whose expression can antagonize mitochondrial Bcl-2 proteins, thus promoting mitochondrial translocation and multimerization of Bax [Schuler and Green, 2001; Miyashita and Reed, 1995; Yu et al, 2003]. Apoptosis induced by H_2O_2 (1 mM, 24h) in glioma cells was associated with increased p53 protein expression [Datta et al, 2002]. In rat neural AF5 cells, H_2O_2 (800 μM, 24h) increased the protein expression of p53, p53-Ser15 phosphorylation, PUMA, Noxa and Bax [McNeill-Blue et al, 2006].

Mild thermotolerance decreased H_2O_2-induced p53 activation, and induction of the target proteins PUMA and Bax, in HeLa cells. This protective effect could be attributed to both Hsps and antioxidants. However, regulation of the p53 pathway by Hsps is complex and not well understood. Hsp70 is involved in suppression of p53-induced senescence signaling in transformed cells, which indicates that Hsp70 plays an essential role in maintaining cell growth upon activation of oncogenic signalling pathways [Gabai

et al, 2009]. Pre-treatment with thermal stress (42°C, 4h) protected HepG2 cells from UVC-induced apoptosis [Chen et al, 1999b]. The anti-apoptotic effect of thermal stress appeared to be due to formation of a HSP70-p53 complex, thus allowing increased stability and DNA binding activity of p53. Consequently, p53 could perform its anti-apoptotic effect by activating p53-dependent genes such as p21 and GADD45 that are involved in DNA repair. Hsp70, in co-operation with Hsp40, can inhibit mitochondrial Bax translocation [Beere, 2004]. Interactions between Hsps and PUMA have not been reported.

Interactions between p53, ROS and antioxidants are complex and not well understood [Liu et al, 2008]. p53 is able to regulate apoptosis by protein interactions at mitochondria and at the transcriptional level in the nucleus [Slee et al, 2004]. During apoptosis induced by tumor promoter 12-O tetradecanoylphorbol-13-acetate, p53 underwent translocation to mitochondria, where it directly bound to and inhibited MnSOD [Zhao et al, 2005]. p53-mediated MnSOD inactivation led to subsequent activation of p53 transcriptional activity and induction of pro-apoptotic target genes such as *Bax*. Translocation of p53 to the nucleus and induction of Bax protein was blocked by a SOD mimetic ($MnTE\text{-}2\text{-}PyP^{5+}$). This indicates that overexpression of MnSOD during mild thermotolerance could inhibit H_2O_2-mediated activation of p53. Furthermore, overexpression of catalase protected HepG2 cells against apoptosis induced by DNA-damaging agents [Bai and Cederbaum, 2003]. The protective effect was associated with decreased p53 phosphorylation and accelerated proteasomal degradation of p53 protein. p53 is a redox-sensitive protein that undergoes oxidation at cysteine residues that contain redox-sensitive thiol groups [Liu et al, 2008]. During oxidative stress, p53 undergoes S-glutathionylation, which inhibits DNA binding activity of p53. This process was reversed when GSH levels were increased by N-acetylcysteine. During mild thermotolerance, increased cellular levels of glutathione could also affect the S-glutathionylation status and function of p53. Furthermore, increased binding of p53 to GSH could occur, which would prevent p53

binding and inactivation of MnSOD, thus maintaining mitochondrial antioxidant activity. It appears that each of the antioxidants that are increased during mild thermotolerance (40°C) is capable of regulating the pro-apoptotic activity of p53.

CONCLUSION

This study shows that febrile temperatures such as 40°C can induce mild thermotolerance and upregulation of the expression of antioxidants, in addition to Hsps. Preconditioning with mild heat stress is an adaptive stress response that could protect cells and tissues against damage caused by diverse environmental stresses (e.g. pesticides, radiation, pharmaceutical drugs and hypoxia) whose toxicity is mediated by ROS. Adaptive stress responses favour survival responses by counteracting stress-induced damage to lipids, proteins and DNA, increasing cellular tolerance to such damage, or by inhibiting pro-apoptotic signaling pathways (e.g. p53). If the stress is too severe, then damaged cells are eliminated by apoptosis. Improved knowledge about mechanisms of adaptation to stressful and extreme environments provides the basis for understanding environmental health problems, performing toxicological risk assessment, and in clinical applications against many disease states that involve ROS, such as neurodegenerative and respiratory disorders, atherosclerosis and cancer.

ACKNOWLEDGMENTS

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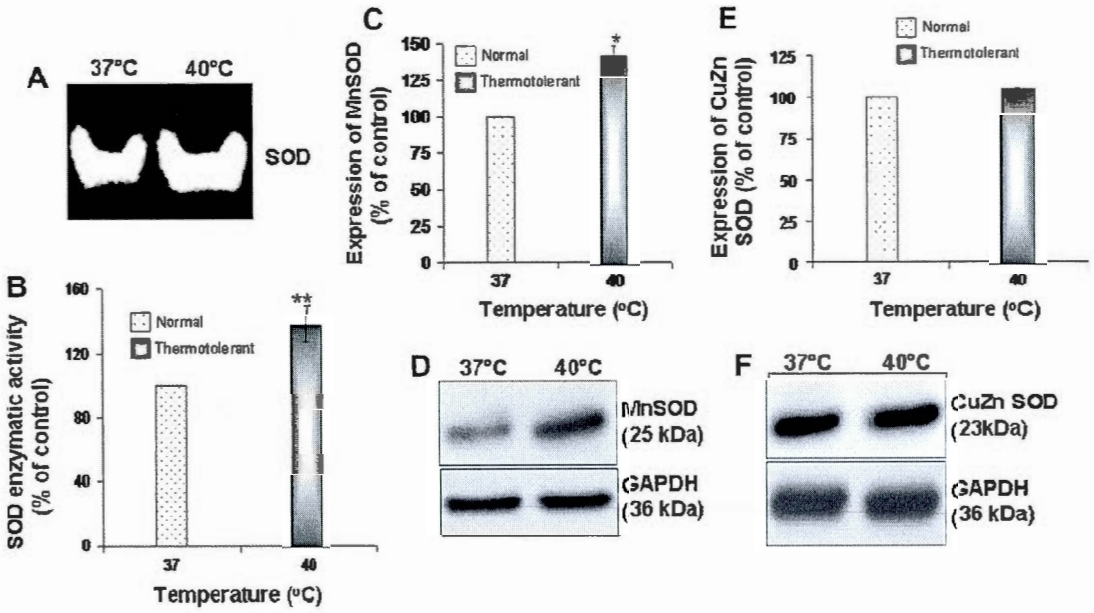


Figure 1 A mild temperature of 40 °C induces enzymatic activity and protein expression of antioxidant MnSOD. (A) Enzymatic activity of SOD was analysed by zymography. SOD activity in thermotolerant (3h, 40°C) (□) and non-thermotolerant (3h, 37°C) (□) HeLa cells was revealed as discoloured bands on a purple colored background after staining with NBT. (B) Enzymatic activity was quantified by densitometry relative to non-thermotolerant control cells at 37°C (100 %). (C-F) Protein expression of MnSOD and CuZnSOD was detected by Western blot using GAPDH (36 kDa) as loading control. Representative blots from three independent experiments are shown for expression of MnSOD (25 kDa) (D) and CuZnSOD (23 kDa) (F). Protein levels of MnSOD (C), and CuZnSOD (E) were analysed by densitometry and expressed relative to control cells at 37°C (100%). For levels/activity of SOD at 40°C relative to controls at 37°C: $P<0.05$ (*), $P<0.01$ (**).

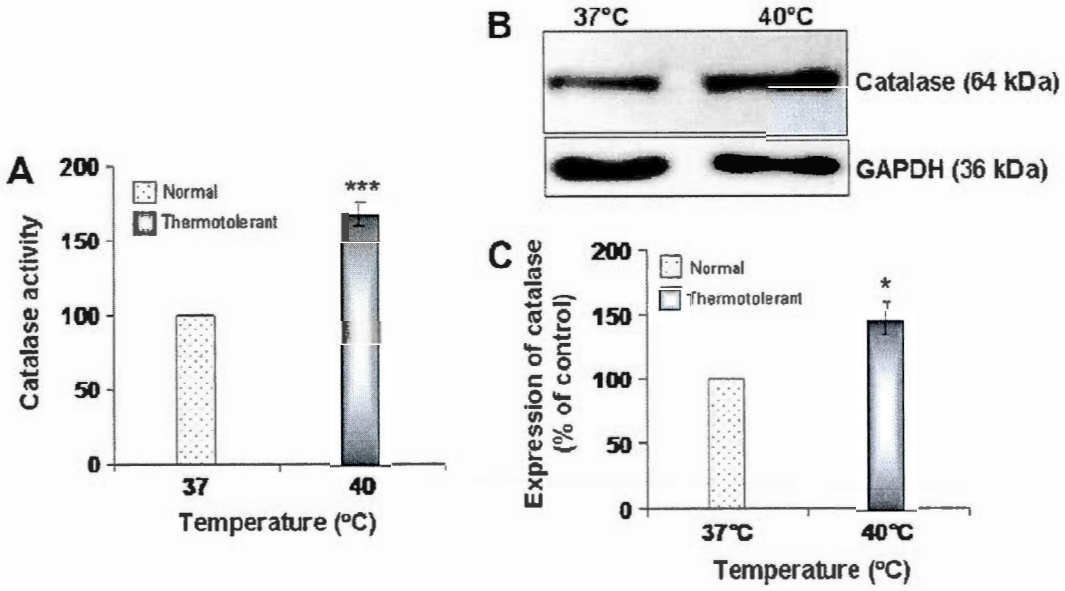


Figure 2 Mild heat treatment at 40 °C increases activity and protein expression of H_2O_2 scavenger catalase. (A) Enzymatic activity and (B, C) protein expression of catalase were analysed in whole cell lysates of thermotolerant (3h, 40°C) (◻) and non-thermotolerant (3h, 37°C) (⊡) HeLa cells. (A) Catalase activity increased from 3.71 ± 0.67 to 6.20 ± 0.30 µmol/min/10^6 cells. Mean and SEM from three independent experiments. (B) Representative blots for expression of catalase are from three independent experiments. (C) Densitometric analyses of catalase expression in thermotolerant cells are relative to non-thermotolerant control (37°C) cells (100%). For levels/activity of catalase at 40°C relative to controls at 37°C: $P<0.05$ (*), $P<0.01$ (**).

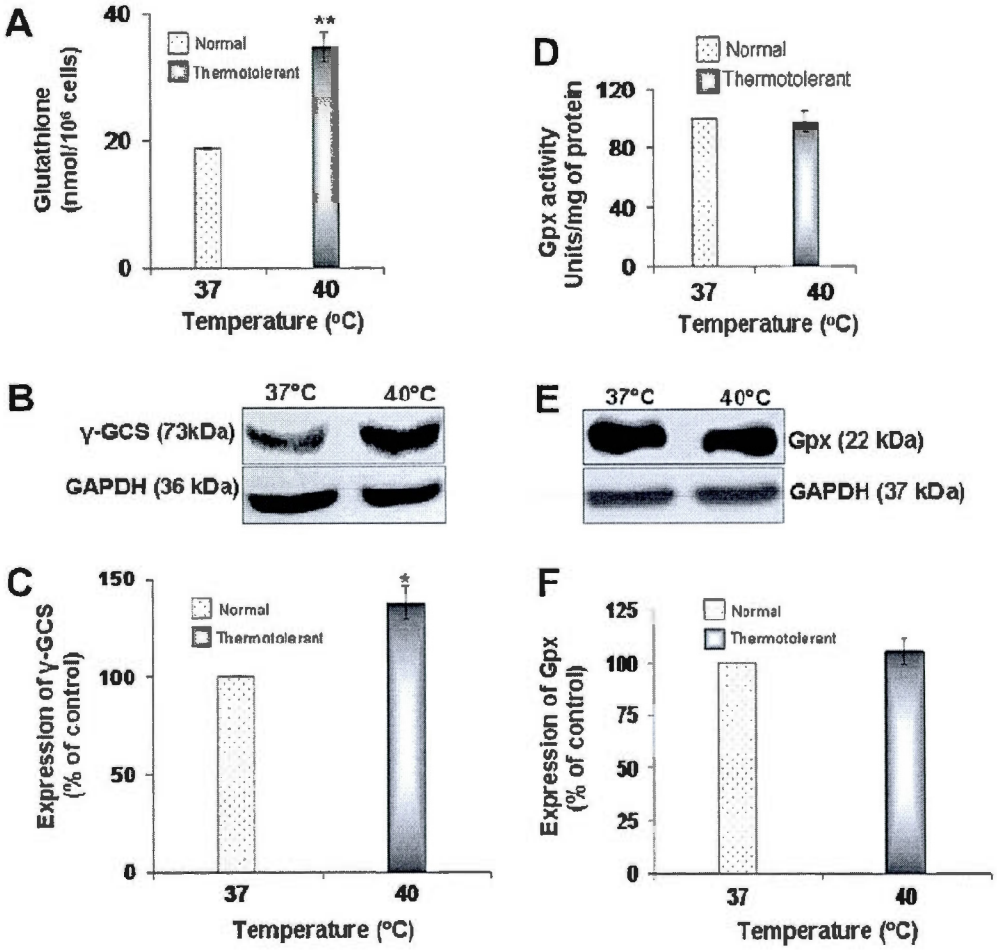


Figure 3 Mild thermotolerance at 40 °C increases intracellular levels of antioxidant glutathione and γGCS. (A) Intracellular glutathione content and (D) GPx activity were determined in thermotolerant (3h, 40°C) (▣) and non-thermotolerant (3h, 37°C) (⊡) HeLa cells. Representative blots from three independent experiments are shown for protein expression of (B) γGCS (73 kDa) and (E) GPx (22 kDa), using GAPDH (37kDa) as loading control. Densitometric analyses of the expression of (C) γGCS and (F) Gpx are relative to non-thermotolerant control (37°C) cells, designated as 100%. For glutathione and γGCS levels at 40C relative to controls at 37°C: $P<0.05$ (*), $P<0.01$ (**).

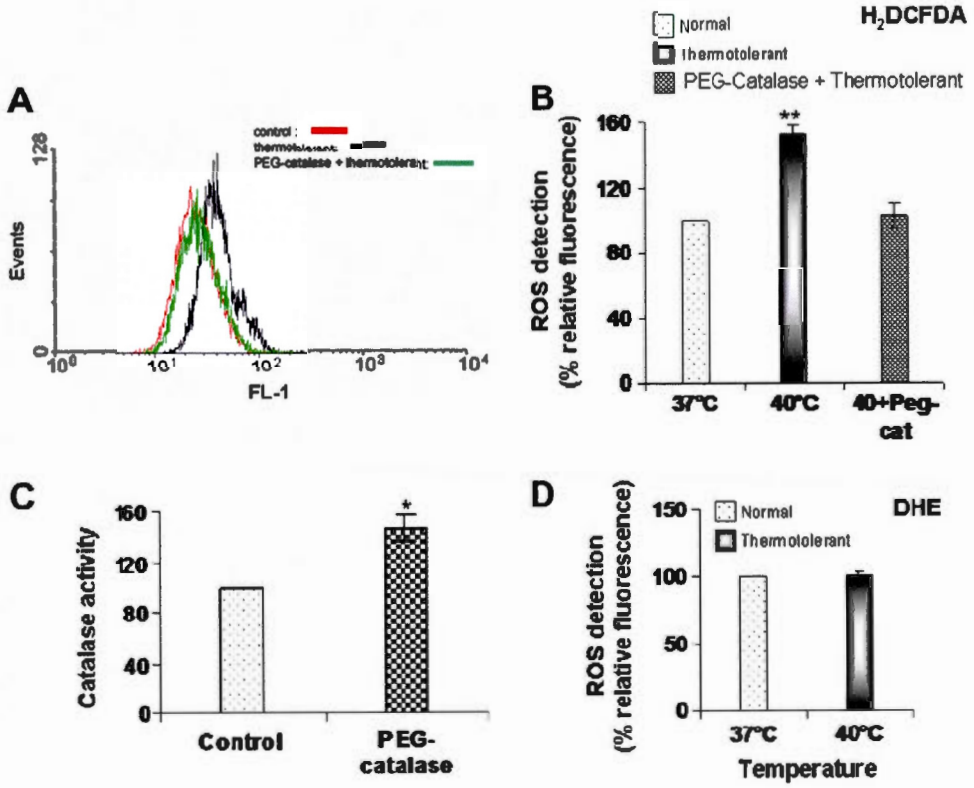


Figure 4 ROS generation increases during induction of mild thermotolerance at 40 °C. (A) A representative FacsScan plot from three independent experiments is shown for H_2DCFDA fluorescence in thermotolerant (3h, 40°C) (black line) and non-thermotolerant (3h, 37°C) (red line) HeLa cells. Where indicated, thermotolerant cells were pretreated with 300 U/ml PEG-catalase for 3h (green line). Data for relative fluorescence intensity for (B) H_2DCFDA and (D) DHE represent means and SEM from three independent experiments and are relative to the untreated control (100%). (C) Catalase activity was determined in cells (5 x 10^6) following incubation with 300 U/ml PEG-catalase for 3h, relative to controls. For catalase activity and ROS levels at 40°C relative to controls at 37°C: $P<0.05$ (*), $P<0.01$ (**).

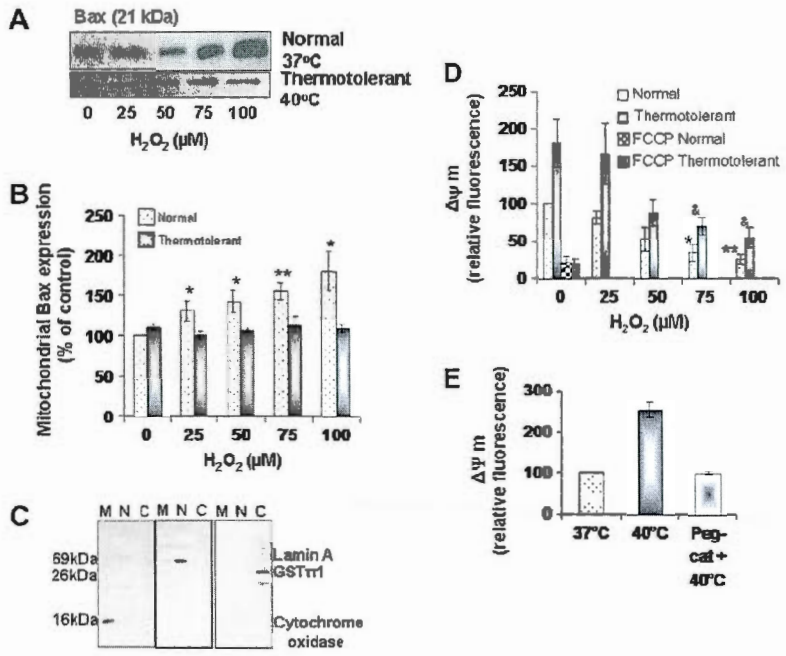


Figure 5 Thermotolerance developed at 40 °C protects cells against H_2O_2-induced mitochondrial Bax translocation and mitochondrial membrane depolarization. Thermotolerant (3h, 40°C) (▣) and non-thermotolerant (3h, 37°C) (⊡) HeLa cells were incubated for 60 min at 37°C with H_2O_2 (0-100 μM) in D-MEM containing 10% FBS. (A) Representative blots are shown for Bax (21 kDa) protein levels in mitochondrial fractions. (B) Densitometric analysis of mitochondrial Bax expression is relative to non-thermotolerant control cells at 37°C (100%). Means and SEM are from four independent experiments. (C) A representative blot is shown for purity of subcellular fractions, using lamin A, glutathione S-transferase (GST-π1) and cytochrome oxidase for nuclear (N), cytosolic (C) and mitochondrial (M) fractions, respectively. (D) Thermotolerant (▣) and non-thermotolerant (⊡) control cells at 37°C (100%) were analyzed by flow cytometry for JC-1 fluorescence, (E) with or without PEG-catalase. FCCP (25 μM) was added to non-thermotolerant (⊠) and thermotolerant (▩) cells as a positive control for mitochondrial membrane depolarization. Data represent means and SEM of relative fluorescence intensity from three independent experiments. $P<0.05$ (*, &) or $P<0.01$ (**, &&) indicates a statistically significant difference between treatment with H_2O_2 and the respective untreated control, for thermotolerant (&) or non-thermotolerant (*) cells. (B,C) For comparison of curves for Bax expression and membrane potential in thermotolerant versus normal cells, $P<0.0001$.

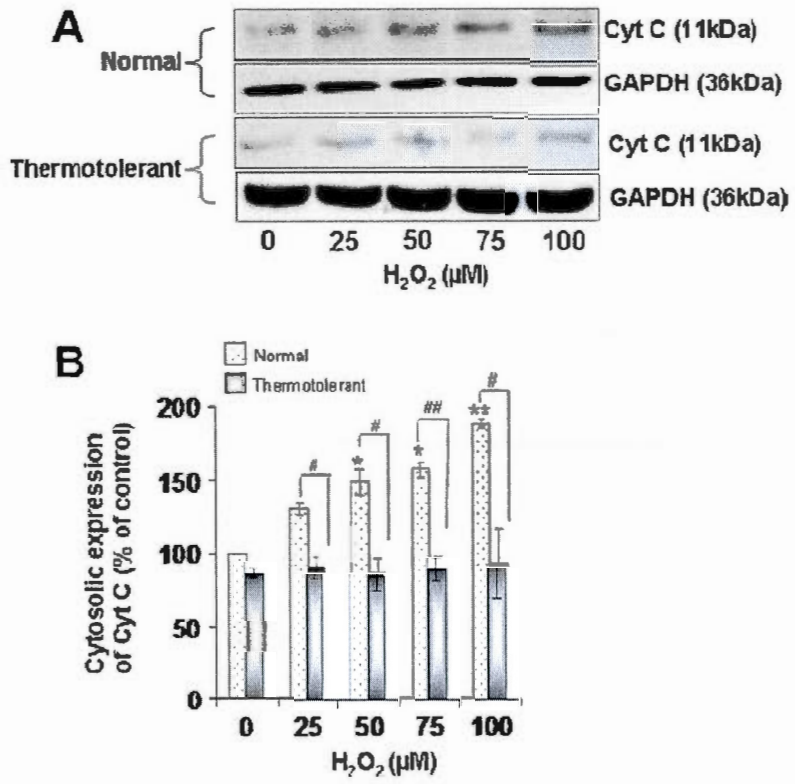


Figure 6 H_2O_2-induced release of cytochrome c from mitochondria is decreased by mild thermotolerance (40 °C). Thermotolerant (3 h, 40°C) (□) and non-thermotolerant (3h, 37°C) (□) HeLa cells were incubated with H_2O_2 (0-100 μM) at 37°C for 2h. (A) Representative blots are shown for cytosolic expression of cytochrome c (11 kDa) using GAPDH (37 kDa) as loading control. (B) Protein levels were analysed by densitometry and expressed relative to control cells at 37°C (100%). Data are mean and SEM of three independent experiments. $P<0.05$ (*, &) or $P<0.01$ (**, &&) indicates a statistically significant difference between treatment with H_2O_2 and the respective untreated control, for thermotolerant (&) or non-thermotolerant (*) cells. $P<0.05$ (#), $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference for cytochrome c levels between thermotolerant and non-thermotolerant cells.

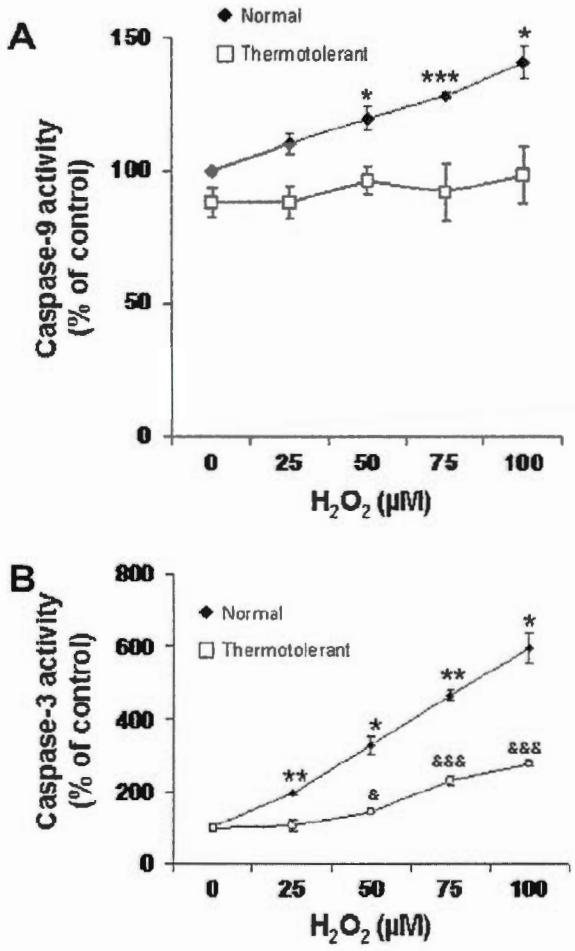


Figure 7 Mild thermotolerance (40 °C) inhibits activation of caspase-9 and caspase-3 by H_2O_2. Thermotolerant (3 h, 40°C) (▣) and non-thermotolerant (3h, 37°C) (⊡) HeLa cells were incubated with H_2O_2 (0-100 μM) at 37°C for 3h. Enzymatic activities of (A) caspase-9 and (B) caspase-3 were determined by spectrofluorimetry and expressed relative to non-thermotolerant control (37°C) cells, designated as 100%. Data represent mean and SEM from three independent experiments performed with multiple estimations per point. $P<0.05$ (*, &), or $P<0.01$ (**, &&), or $P<0.001$ (***) indicates a statistically significant difference for +/- H_2O_2, for thermotolerant or non-thermtolerant cells. For comparison of curves for caspase-9 and caspase-3 activity in thermotolerant versus non-thermotolerant cells, $P<0.0001$ and $P=0.0004$, respectively.

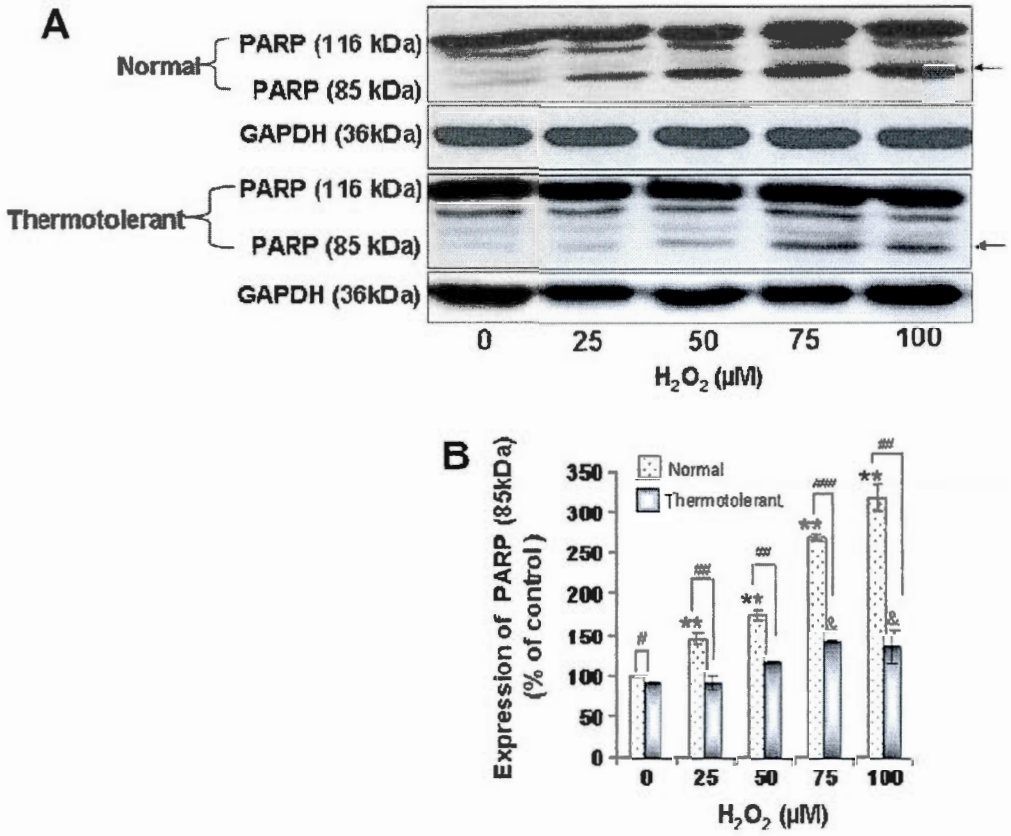


Figure 8 Mild thermotolerance diminishes H_2O_2-induced PARP cleavage. Thermotolerant (3h, 40°C) (▫) and non-thermotolerant (3h, 37°C) (▫) HeLa cells were incubated for 3h at 37°C with H_2O_2 (0-100 μM) in D-MEM containing 10% FBS. (A) Immunodetection of native PARP (116 kDa) and cleaved PARP (85 kDa) in whole cell lysates was carried out by Western blotting, using GAPDH (37 kDa) as loading control. (B) Densitometric analysis of cleaved PARP expression is relative to non-thermotolerant control (37°C) cells, designated as 100%. Data represent means and SEM from four independent experiments performed with multiple estimations per point. $P<0.05$ (*, &) or $P<0.01$ (**, &&) indicates a statistically significant difference between treatment with H_2O_2 and the respective untreated control, for thermotolerant (&) or non-thermotolerant (*) cells. $P<0.05$ (#), $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference for cleaved PARP between normal and thermotolerant cells.

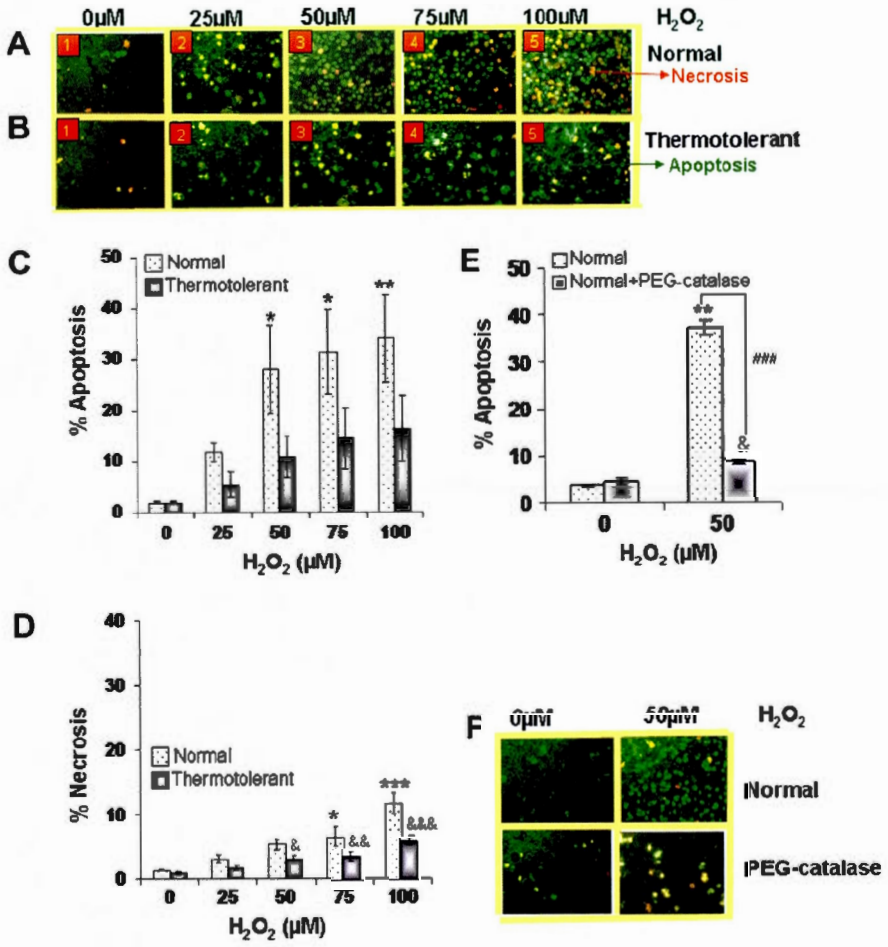


Figure 9 Mild thermotolerance at 40 °C protects against H_2O_2-induced chromatin condensation and necrosis. Thermotolerant (3h, 40°C (B1-B5)) and non-thermotolerant (3h, 37°C (A1-A5)) HeLa cells were incubated with H_2O_2 (0-100 μM) for 3h. (E, F) Cells were pre-incubated for 3h with 300 U/ml of PEG-catalase before addition of 50 μM H_2O_2. Nuclear morphology was assessed by fluorescence microscopy using Hoechst 33258 to detect blue-green apoptotic cells and PI to detect red necrotic cells (Magnification 320X). The fractions of apoptotic (C, E) and necrotic (D) cells are relative to total cells. (A, B, F) Photographs are representative of three individual experiments. $P<0.05$ (*, &), $P<0.01$ (**, &&), or $P<0.001$ (***, &&&) indicates a statistically significant difference between treatment with H_2O_2 and the respective control. (E) $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference for apoptosis in cells +/- PEG-catalase.

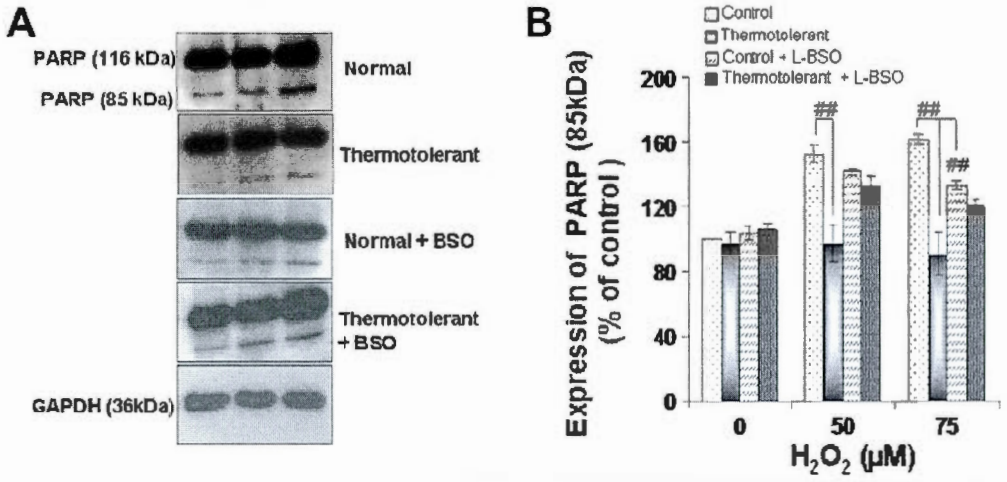


Figure 10 Inhibition of glutathione synthesis during thermotolerance partially decreases the development of resistance to H_2O_2-induced PARP cleavage. HeLa cells were pretreated for 3h at 37°C (non-thermotolerant) or 40°C (thermotolerant), with or without BSO (1mM), and then exposed to H_2O_2 (0, 50, 75μM) for 3h at 37°C in D-MEM containing 10% FBS. A) Immunodetection of native PARP (116 kDa) and cleaved PARP (85 kDa) in whole cell lysates was carried out by Western blotting, using GAPDH (37 kDa) as loading control. (B) Densitometric analysis of cleaved PARP expression is relative to non-thermotolerant controls (37°C), designated as 100%. Data represent means and SEM from four independent experiments performed with multiple estimations per point. $P<0.01$ (##) indicates a statistically significant difference for cleaved PARP between normal and thermotolerant cells, or normal versus L-BSO treated cells.

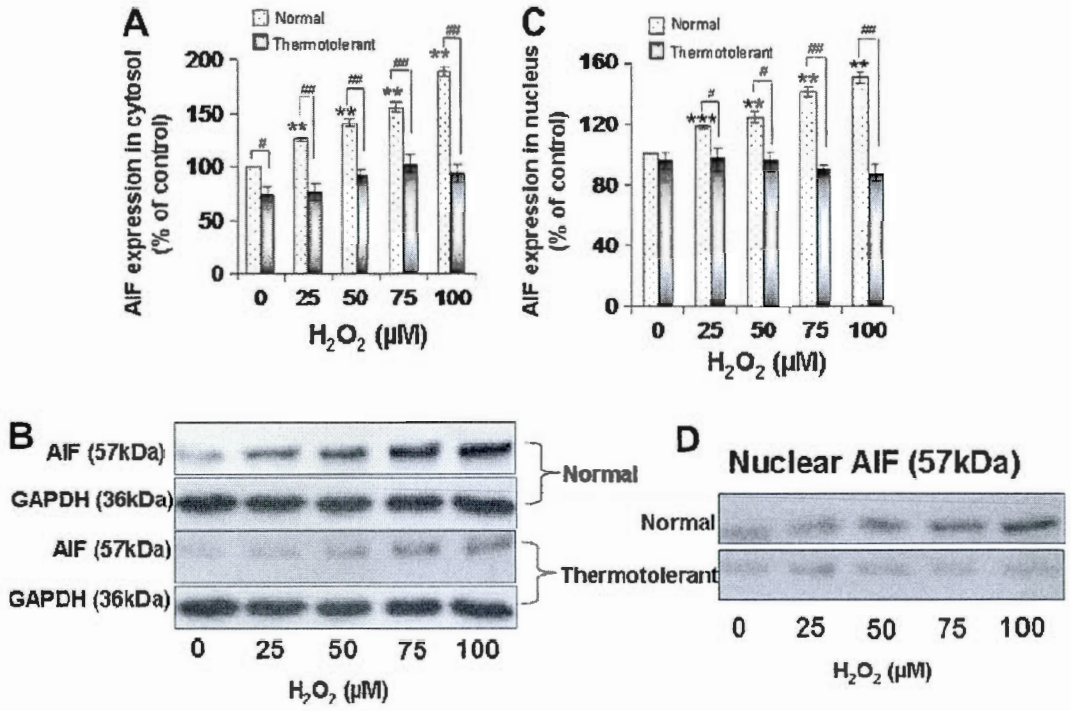


Figure 21 Thermotolerance induced at 40 °C inhibits H_2O_2-induced translocation of AIF from mitochondria to the nucleus. Thermotolerant (3h, 40°C) (◻) and non-thermotolerant (3h, 37°C) (⊡) HeLa cells were incubated for 1h at 37°C with H_2O_2 (0-100 μM) in D-MEM containing 10% FBS. Representative blots for expression of cytosolic (B), mitochondrial (data not shown), and nuclear (D) AIF (57 kDa) are shown, using GAPDH (37 kDa) as loading control for the cytosolic fraction. Densitometric analyses of the expression of (A) cytosolic AIF and (C) nuclear AIF are shown relative to control cells at 37°C (100%). Data represents mean and SEM from three independent experiments. $P<0.05$ (*, &) indicates a statistically significant difference between treatment with H_2O_2 and the respective untreated control. $P<0.05$ (#) or $P<0.01$ (##) indicates a statistically significant difference for AIF between normal and thermotolerant cells.

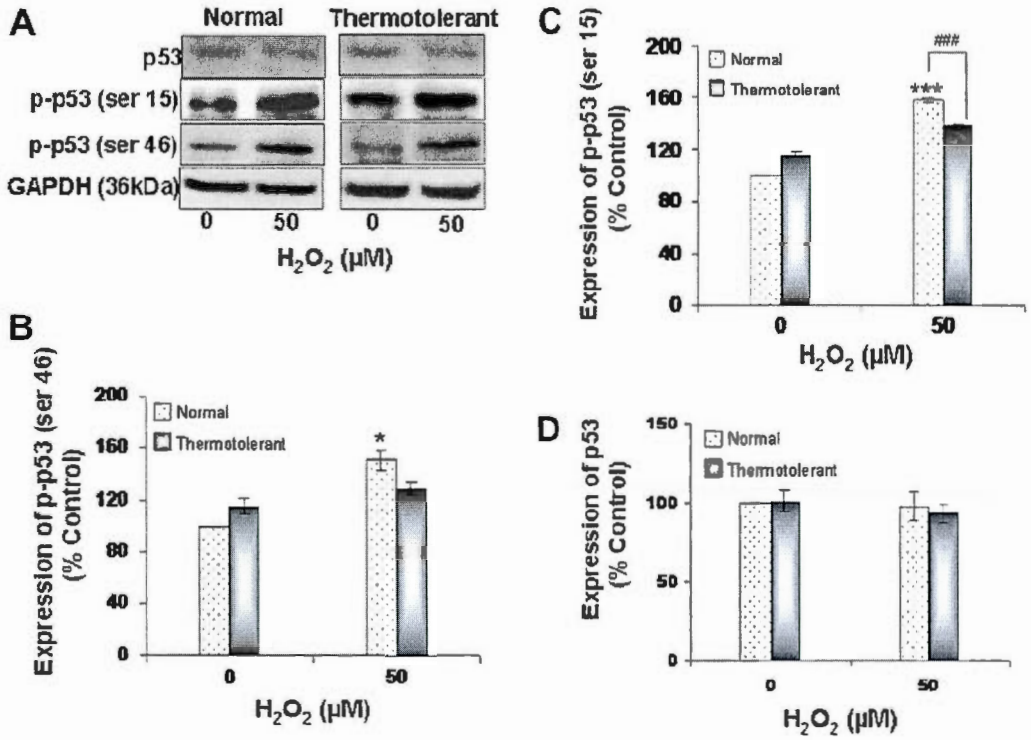


Figure 3 H_2O_2-induces phosphorylation of p53 at ser 15 and ser 46. Thermotolerant (3h, 40°C) (▣) and non-thermotolerant (3h, 37°C) (□) HeLa cells were incubated with H_2O_2 (0, 50 μM) for 1h and then immunodetection of p53, p-p53 (Ser15), and p-p53 (Ser46) was carried out. (A) Representative blots are shown from three independent experiments. Densitometric analyses of the expression of p53 (D), p-p53 (Ser46) (B), and p-p53 (Ser15) (C) are relative to non-thermotolerant control (37°C) cells, designated as 100%, using GAPDH as loading control. Data represents mean and SEM from three independent experiments performed with multiple estimations per point. $P<0.05$ (*, &) or $P<0.001$ (***, &&&) indicates a statistically significant difference between treatment with H_2O_2 and the respective control. $P<0.001$ (###) indicates a statistically significant difference for p53 between normal and thermotolerant cells.

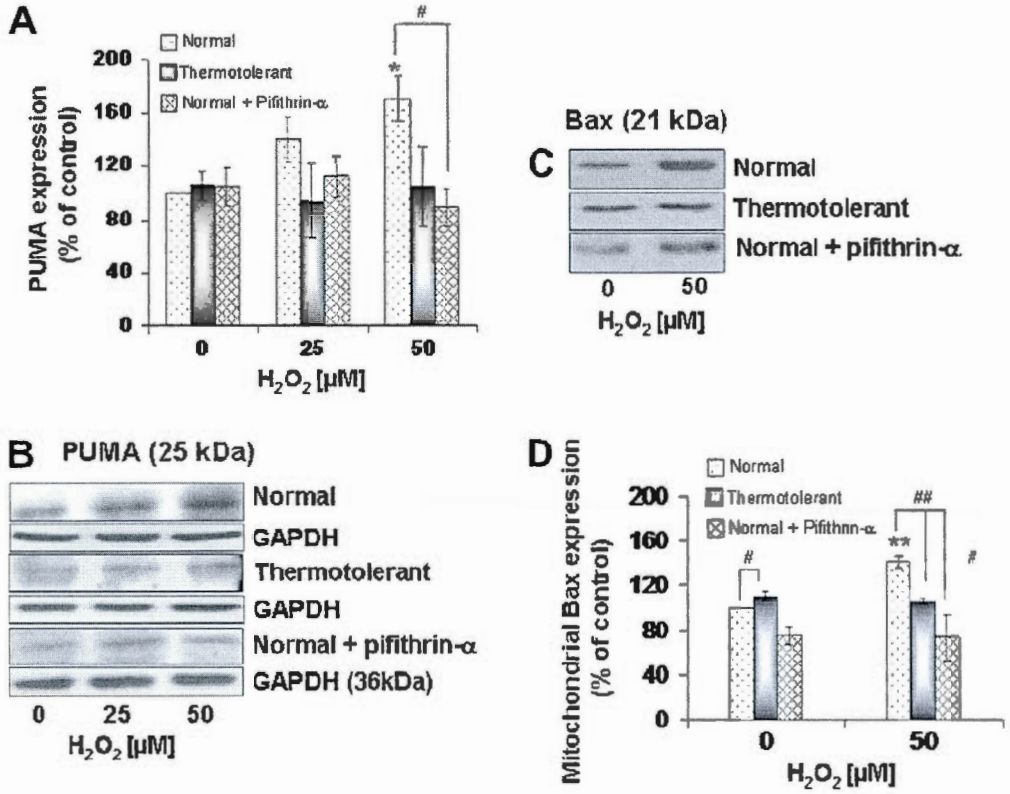


Figure 13 Inhibition of p53 by pifithrin-α decreases induction of PUMA and Bax translocation by H_2O_2. Normal and thermotolerant cells were pretreated for 3h at 37°C with or without 10 μM pifithrin-α and then exposed to H_2O_2 (0-50 μM) for 1h. Representative blots from three individual experiments are shown for expression of (B) PUMA (25 kDa) and (C) Bax (21 kDa) in mitochondrial fractions, with or without pifithrin-α. Densitometric analyses of (A) PUMA and (D) Bax expression are shown relative to control cells (100%). Data represents mean and SEM from five independent experiments. $P<0.05$ (*) or $P<0.01$ (**) indicates a statistically significant difference between treatment with H_2O_2 and the untreated control. $P<0.05$ (#) or $P<0.01$ (##) indicates a statistically significant difference between cells exposed to H_2O_2, with and without pifithrin-α.

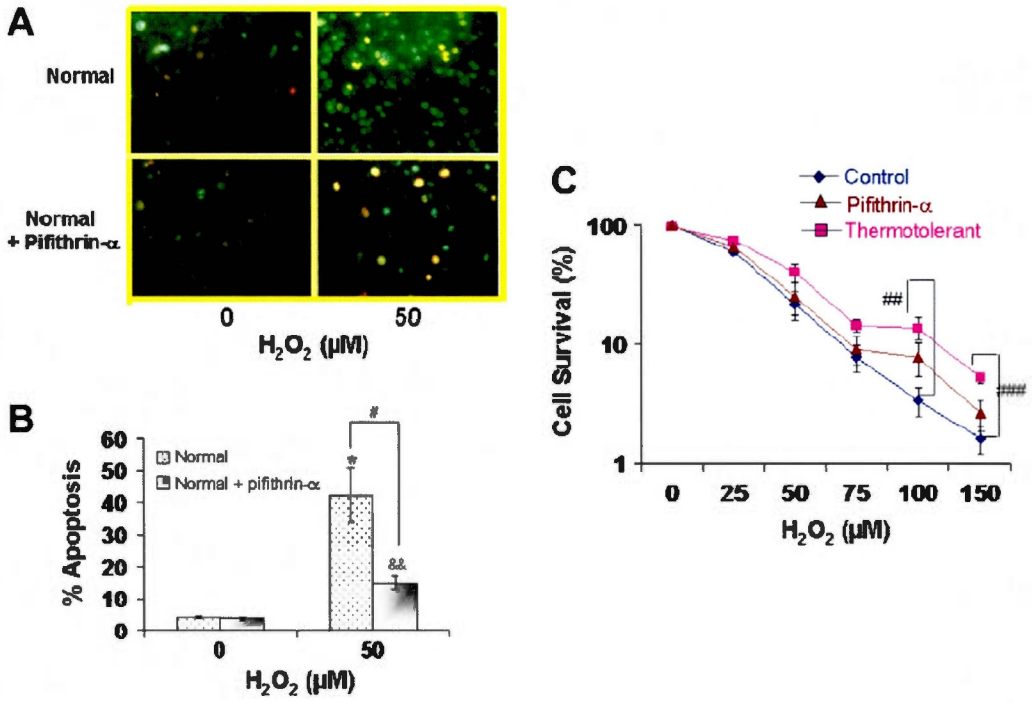
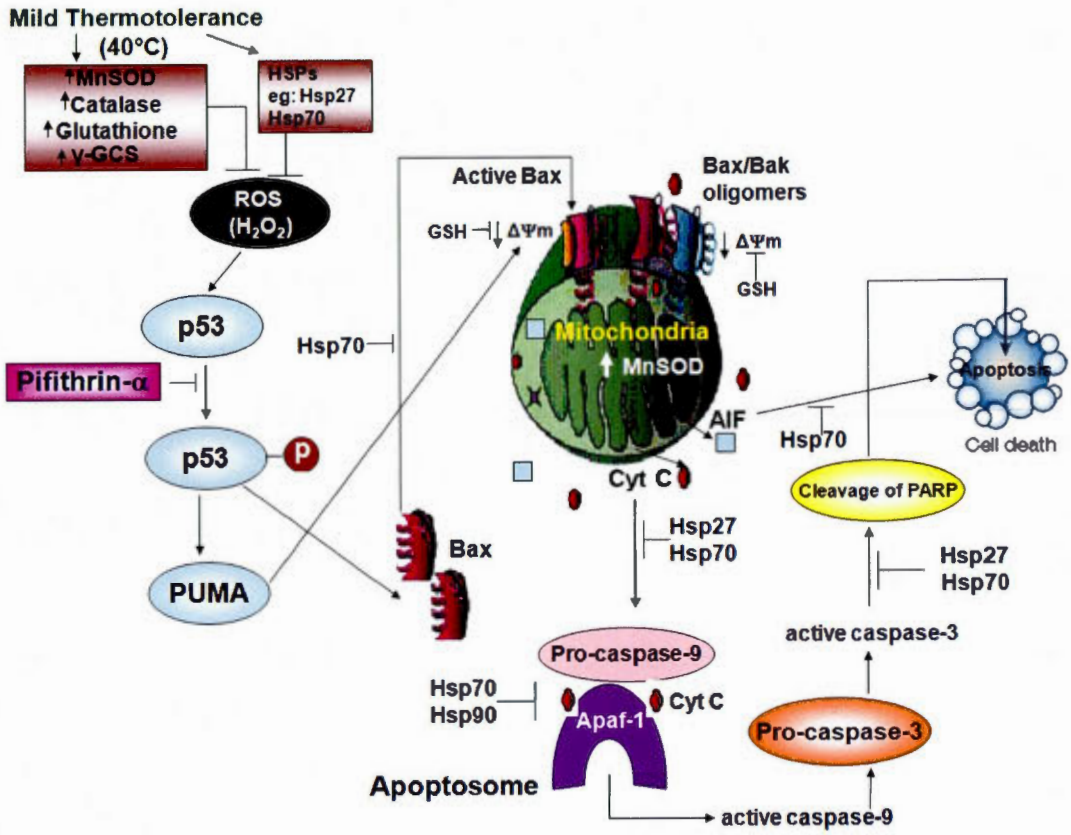


Figure 14 Effect of pifithrin-α on induction of chromatin condensation and cytotoxicity by H_2O_2. (A) Chromatin condensation was visualized using Hoechst 33258. (B) The fractions of apoptotic cells, with or without pifithrin-α, are given relative to total cells. Data represents mean and SEM from four (C) or five (A, B) independent experiments. P<0.05 (*, &) or P<0.01 (**, &&) indicates a statistically significant difference between treatment with H_2O_2 and the respective untreated control, for thermotolerant (&) or non-thermotolerant (*) cells. P<0.05 (#) indicates a statistically significant difference between cells exposed to H_2O_2, with and without pifithrin-α. (C) Cytotoxicity of H_2O_2 in thermotolerant versus normal cells was assessed by a clonogenic cell survival assay. Where indicated, cells were pretreated with 10 μM pifithrin-α. The control value represents 10^5 cells and was normalized to represent 100% cell survival. P<0.01 (##) or P<0.001 (###) indicates a statistically significant difference between cells exposed to H_2O_2, for thermotolerant or non-thermotolerant cells. For curve analysis of thermotolerant versus non-thermotolerant cells, p = 0.0002



Scheme 1 Mild thermotolerance protects against induction of mitochondrial apoptosis by pro-oxidant H_2O_2.

2.3 ARTICLE II

MILD THERMOTOLERANCE INDUCED AT 40°C PROTECTS HELA CELLS AGAINST ACTIVATION OF DEATH RECEPTOR-MEDIATED APOPTOSIS BY HYDROGEN PEROXIDE

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RÉSUMÉ

L'exposition des cellules à des températures modérées de 40°C induit une thermotolérance leur permettant de résister à des expositions toxiques subséquentes. Cette recherche étudie les mécanismes impliqués dans les effets protecteurs d'une thermotolérance modérée (40°C, 3h) contre l'activation de la voie extrinsèque de l'apoptose induite par le peroxyde d'hydrogène (H_2O_2) chez les cellules HeLa. Le peroxyde d'hydrogène (5-50 μM) entraîne une activation rapide (1-3h) de la voie des récepteurs de la mort activée par Fas, ce qui est mis en évidence par une régulation positive de FasL et le recrutement de sa molécule adaptatrice FADD (Fas Associated Death Domain) au niveau de la membrane plasmique. Ceci résulte en l'activation de la caspase 8 et de la caspase 2, lesquelles conduisent à l'activation de la voie impliquant le clivage de Bid, la translocation de tBid à la mitochondrie et l'activation de la caspase 9. Les cellules thermotolérantes montrent toutes une diminution de ces effets. La thermotolérance modérée protège à la fois contre la cytotoxicité du peroxyde d'hydrogène (H_2O_2), l'activation de la caspase 3 et la condensation de la chromatine. L'antioxydant PEG-catalase (polyéthylène glycol catalase) inhibe l'induction de FasL et l'activation de la caspase 8 causées par le peroxyde d'hydrogène. La régulation positive de FasL, l'activation des caspases 2-3-8-9 et la condensation de la chromatine ont toutes été diminuées par la présence d'un inhibiteur de p53, la pifithrine-α, impliquant que p53 agit comme un facteur en amont dans l'activation de l'apoptose médiée par la voie des récepteurs de la mort en présence de peroxyde d'hydrogène. Cette étude propose un avancement des connaissances à propos des effets protecteurs aux réponses adaptatives induites par des stress modérées, telle que la température causée par la fièvre, contre l'induction de l'apoptose liée au stress oxydatif.

Mots clé : stress oxydatif, peroxyde d'hydrogène, apoptose, thermotolérance, réponse adaptative, récepteur de la mort, protéine p53, protéines de choc thermique, antioxydant, radicaux libres.

ABSTRACT

Preexposure to mild temperatures such as 40 °C induces thermotolerance, whereby cells resist subsequent exposure to a toxic insult. This study investigates the protective effect of mild thermotolerance (3h, 40 °C) against activation of death receptor-mediated apoptosis by H_2O_2 in HeLa cells. H_2O_2 (5-50 μM) caused rapid activation (1-3h) of the Fas death receptor pathway of apoptosis, which was evident by up-regulation of death ligand FasL and recruitment of adaptor protein Fas-associated death domain to the plasmamembrane. This resulted in activation of caspase-8 and caspase-2, which led to activation of the cross-talk pathway involving Bid cleavage, tBid translocation to mitochondria and caspase-9 activation. These changes were all diminished in thermotolerant cells. Mild thermotolerance also protected cells against cytotoxicity of H_2O_2 as well as execution phase events of apoptosis such as caspase-3 activation and chromatin condensation. The antioxidant polyethylene glycol-catalase abolished FasL induction and caspase-8 activation due to H_2O_2. FasL up-regulation; activation of caspases-8, -2, -9 and -3 activation; andchromatin condensation were decreased by p53 inhibitor pifithrin-α, implicating p53 as an upstream factor in activation of death receptor-mediated apoptosis by H_2O_2. This study advances knowledge about the protective effect of adaptive responses induced by mild stresses, such as fever temperatures, against induction of apoptosis by oxidative stress.

Key words: Oxidative stress, Hydrogen peroxide, Apoptosis, Thermotolerance, Adaptive response, Death receptor, p53 protein, Caspase, Heat shock protein, Antioxidant, Free radicals.

INTRODUCTION

Reactive oxygen species (ROS) are involved in various normal cellular functions such as proliferation, signalling pathways and apoptosis [Circu and Aw, 2010]. Cells and tissues are equipped with a wide range of antioxidant defense systems that approximately balance the production of ROS under normal conditions [Halliwell and Gutteridge, 2007]. Superoxide, for example, is catalytically removed by superoxide dismutase (SOD) and hydrogen peroxide (H_2O_2) by catalase and glutathione peroxidase. However, the generation of ROS can be increased by various conditions of stress, including ionising radiation, chemotherapeutic drugs, environmental pollutants and herbicides. Increased levels of ROS in cells and tissues can cause an imbalance in the redox equilibrium between pro-oxidants and antioxidants [Halliwell and Gutteridge, 2007; Chen et al, 2007]. A redox imbalance can lead to oxidative damage to essential macromolecules such as proteins, lipids and nucleic acids [Circu and Aw, 2010]. Oxidative cellular damage can result in senescence, in which cells are no longer able to divide, or in cell death processes such as apoptosis and necrosis. Milder conditions of stress generally lead to apoptotic cell death, whereas more severe stress leads to cell death by necrosis [Kroemer et al, 1998].

Apoptosis is a highly regulated cell death process, which plays an important role during embryonic development and tissue homeostasis, by removing excess, infected, transformed or damaged cells [Roya and Mauro, 2004]. Deregulation of this process can lead to development of various pathophysiological conditions, including cancer and neurodegenerative disorders. Characteristic hallmarks of apoptosis include exposure of phosphatidylserine at the extracellular surface, DNA fragmentation, cytoplasmic and nuclear condensation, and the formation of membrane-bound apoptotic bodies that are phagocytosed by macrophages [Elmore, 2007]. The apoptotic process can take place by three different pathways that involve death receptors (extrinsic), mitochondria (intrinsic) and the endoplasmic reticulum (intrinsic). Induction of apoptosis via death receptors such

as Fas receptor (Fas), tumor necrosis factor receptor (TNFR) and TNF-related apoptosis-inducing ligand (TRAIL) [Lavrik et al, 2005] results in the activation of initiator caspases such as caspase-8 or caspase-10, which in turn leads to activation of the effector caspases -3, -6 and -7. The effector caspases are responsible for the cleavage of key cellular proteins, leading to the typical morphological changes observed in apoptotic cells. The death receptor pathway can be interconnected with the mitochondrial pathway through a crosstalk pathway mediated by cleavage of Bcl-2 family protein Bid [Strasser et al, 2009].

Hyperthermia (42-47°C) is among the recent and innovative methods of cancer treatment [Horsman and Overgaard, 2007] and is mainly used as an adjuvant to radiation and chemotherapy. A major advantage is that localised heating allows increased cytotoxicity and the targeting of radiation and chemotherapy treatments to the tumor region. Indeed, hyperthermia is one of the most effective radiation sensitizers known and can eliminate radioresistant tumor cells. Significant improvements in clinical outcome were reported using a combination of hyperthermia with radiotherapy, chemotherapy, or both, for tumours of the head and neck, breast, brain, bladder, cervix, rectum, lung, oesophagus, for melanoma and sarcoma [Van der Zee, 2002; Issels et al, 2006; Van der Zee and Erasmus, 2007]. Despite such promising progress at the clinical level, the mechanisms involved in hyperthermia-induced cell death are not clearly understood [Milleron and Bratton, 2007].

The exposure of cells and tissues to elevated temperatures (39-45°C) induces the expression of heat shock proteins (Hsp) and the phenomenon of thermotolerance [Calderwood et al, 2006; Field and Anderson, 1982; Landry et al, 1989; Przybytkowski et al, 1986]. Thermotolerance is a transient adaptive response where by cells become resistant to a subsequent lethal insult such as that induced by heat shock, oxidative stress, anticancer drugs, radiation and environmental stressors [Gill et al, 1998; Martindale and

Holbrook, 2002; Kregel, 2002]. It usually disappears within several days, and therefore, does not interfere with clinical use of hyperthermia. Thermotolerance can be developed after shorter exposures (e.g. 30 min) to higher, lethal temperatures (42-45°C), or during continuous heating (e.g. 3-24h) at lower, nonlethal temperatures (39.5-41.5°C) [Przybytkowski et al, 1986; Landry et al, 1982]. Most studies have investigated thermotolerance developed at higher, lethal temperatures, whereas thermotolerance developed at mild physiological temperatures (39-41°C), which occur during fevers, is poorly understood.

We reported that mild thermotolerance developed at 40°C increases the production of ROS, as well as the expression/activity of several major antioxidants such as manganese superoxide dismutase (MnSOD), catalase and glutathione as well as its key synthesis enzyme, γ-glutamylcysteine synthetase [Pallepati and Averill-Bates, 2010]. However, the ability of mild thermotolerance to protect cells against the activation of apoptosis is not well understood. This study evaluated the protective role of mild thermotolerance, developed at 40°C, against activation of death receptor-mediated apoptosis by pro-oxidant H_2O_2 in human cervical carcinoma (HeLa) cells. This study also aimed to provide new insights into activation of the death receptor signaling pathway of apoptosis by H_2O_2, including the roles of p53, Fas and caspase-2.

MATERIALS AND METHODS

Cell culture

HeLa cells (ATCC No. CCL-2) were grown in monolayer in Dulbecco's modified Eagle's medium (Invitrogen Canada, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen, Canada), in tissue culture flasks (Sarstedt, St Laurent, QC, Canada) at 37°C in a humidified atmosphere of 5% CO_2 in a water jacketed incubator [Bettaieb and Averill-Bates, 2005]. Cell culture medium was replaced with fresh medium 24h before experiments. To induce thermotolerance, cells were transferred to an identical incubator for 3h at 40°C (± 0.1°C), following a period of 20min to allow the temperature of the culture medium to reach 40°C [Pallepati and Averill-Bates, 2010]. The cells were grown to near confluence and then harvested using 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution, and washed by centrifugation (1000 g, 3 min). There was no loss of cell viability, using trypan blue, in cells heated at 40°C for 3h.

Treatment with H_2O_2 and inhibitors

Confluent cells in monolayer were pretreated, where indicated, with the following inhibitors: Fas antagonist Kp7-6 (1 mM, 1 h), caspase-8 (Z-IETD-FMK, 20 μM, 2 h), caspase-2 (Z-VDVAD-FMK, 25 μM, 1 h), pifithrin-α (10 μM, 1 h) a reversible inhibitor of p53-mediated apoptosis (Calbiochem, La Jolla, CA, USA), or polyethylene glycol-catalase (PEG-catalase) (300 U/ml, 3h) (cell permeable H_2O_2 scavenger) (Sigma-Aldrich Canada). Freshly harvested thermotolerant and non-thermotolerant HeLa cells were then resuspended in D-MEM plus 10% FBS and incubated at constant cell density ($1x10^6$/ml) with 25 to 100 μM of H_2O_2, in a final volume of 1.0 ml at 37°C for 1 or 3 h, along with untreated controls. Cells were washed by centrifugation (1000 g, 3 min) to remove H_2O_2, and then analysed for Fas ligand (FasL) expression, caspase-8, -2, -9 and -3 activation; Bid cleavage; and chromatin condensation.

Flow cytometric analysis of FasL expression

After treatment with H_2O_2, thermotolerant (3 h, 40 °C) and non-thermotolerant (3 h, 37 °C) HeLa cells (10^6) were incubated with 20 μg/ml of (PE)-labelled anti-FasL [Kayagaki et al, 1995] (Caltag Laboratories, Burlingame, CA, USA) for 30 min at 22°C in the dark [Filion et al, 2004]. Cells (10,000) were washed and analysed using a FACScan flow cytometer equipped with an argon laser (Ex. 488 nm). Data were analysed using Cell Quest software (Becton Dickinson, Oxford, UK). The mean fluorescence intensity of 10,000 cells was calculated for each sample and corrected for autofluorescence obtained from samples of unlabeled cells. The extracellular expression of FasL was compared with untreated cells.

Immunodetection of total protein expression

For detection of procaspase-8 and inhibitor of caspase-activated DNase (ICAD), thermotolerant and nonthermotolerant cells were harvested and incubated with H_2O_2 at 37°C for the required times. Cells were washed by centrifugation (1000 g, 3 min) in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) [Samali et al, 1999]. The supernatant was discarded, pelleted cells were resuspended in lysis buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 1h. Then, by a 10 min centrifugation step at 2500 g to remove nuclei and unbroken cells, the proteins of whole cell lysates were isolated in the supernatant [Tanel and Averill-Bates, 2005]. Proteins (30 μg) [Bradford, 1976] were separated by SDS-polyacrylamide gel electrophoresis [Laemmli, 1970] and transferred to polyvinyl difluoride membranes, which were probed with the primary antibodies (1:1000): ICAD, caspase-8 or GAPDH (Santa Cruz Biotechnology Inc.). Secondary antibodies (1:5000) were goat horse-radish peroxidase (HRP)-conjugated polyclonal anti-mouse IgG or HRP-conjugated rat MAb-anti-rabbit IgG (Biosource, Camarillo, CA). Membranes were incubated in ECL-Plus chemiluminescence reagent (Amersham Biosciences Corp, Piscataway, NJ) and films

were scanned with a Laser Scanning Densitometer (Alpha Innotech Corp, San Leandro, CA). Total protein expression was analysed using IPGEL software (BD Biosciences Bioimaging, Rockville, MD) and normalized to GAPDH as loading control.

Immunodetection of proteins in subcellular fractionations

Subcellular fractions were prepared as described previously [Samali et al, 1999; Tanel and Averill-Bates, 2005]. Thermotolerant and nonthermotolerant cells were washed in buffer A and resuspended in buffer B containing 0.1 mM dithiothreitol (DTT). Lysates were homogenised using a Dounce homogeniser (50 strokes/sample). After a 30 min incubation on ice, unbroken cells and nuclei were pelleted by centrifugation at 2500 g for 10 min. The supernantant was centrifuged further at 15,000 g for 15 min. The pellet containing the mitochondrial fraction was then resuspended in buffer C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS, 0.1 mM DTT, 100 μl/10 ml of cocktail of protease inhibitors, pH 7.4) and used for detection of Bid/tBid. The supernatant was further centrifuged at 100 000 g for 1 h. The resulting supernatant (cytosolic fraction) was used for detection of FADD and Bid/tBid. The pellet was resuspended in lysis buffer B and designated as the microsomal fraction, which was used to detect FADD and FasL. Microsomal extracts contain plasma membranes [Gomez-Angelats and Cidlowski, 2001]. Subcellular distribution of proteins (30μg) (FADD, Bid/tBid, FasL, 1:1000) (Santa Cruz Biotechnology Inc.) was analyzed by SDS-PAGE and Western blotting [Bettaieb and Averill-Bates, 2005]. Protein expression was analysed relative to GAPDH using a scanning laser densitometer. Purity of microsomal (92%), cytosolic (98%) and mitochondrial (97%) fractions was determined by Western blotting using calnexin, glutathione S-transferase (GST-π1) and cytochrome oxidase, respectively.

Caspase activity

After treatment with H_2O_2 (0, 25-50 μM), thermotolerant and nonthermotolerant cells were harvested, and cell lysates were prepared at –80°C for 30 min and then incubated with the following caspase substrates (50 μM) [Tanel and Averill-Bates, 2005]: Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC) for caspase-3, Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC) for caspase-9, Z-Ile-Glu-Thr-Asp-AFC (Z-IETD-AFC) for caspase-8, and Z-Val-Asp-Val-Ala-Asp-AFC (Z-VDVAD-AFC) for caspase-2 (Calbiochem). Caspase substrate cleavage to generate AFC (λmax excitation at 400nm, λmax emission at 505nm) or AMC (λmax excitation at 380 nm, λmax emission at 460nm) was measured by spectrofluorimetry (SpectraMax Gemini, Molecular Devices, Sunnyvale, CA) at appropriate excitation and emission wavelengths. Caspase activities are represented as Vmax of the 30 min kinetic reaction.

Morphological analysis of apoptosis

Thermotolerant and nonthermotolerant cells (10^6/ml) were labelled with Hoechst 33258 (50 μg/ml) (blue-green fluorescence) (Sigma Chemical Co.), which binds to condensed nuclear chromatin of apoptotic cells [Tanel and Averill-Bates, 2005]. Cells were washed with PBS and then propidium iodide (PI) (50 μg/ml) was added to visualize necrotic cells (red fluorescence). Observations were made by fluorescence microscopy (model IM, Carl Zeiss Canada Ltd, St. Laurent, QC) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix imaging Inc, Mississauga, ON). Images were analyzed using Northern Eclipse software (Empix Imaging). Cells were classified using the following criteria: (1) live cells with normal nuclei, pale blue/green chromatin with organized structure); (2) apoptotic cells with bright blue /green condensed or fragmented chromatin); (3) necrotic cells (red, enlarged nuclei with smooth normal structure). Fractions of apoptotic or necrotic cells were calculated relative to total cells. For each condition, 300 cells were counted.

Statistics

Data represent means ± SEM from at least three independent experiments performed in duplicate. When not shown, error bars lie within symbols. Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means. The Bonferroni-Holmes adjustment was used to control for the family-wise error rate at a desired level (α=5%). The software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC, USA). For significant differences, $P < 0.05$.

RESULTS

Mild thermotolerance developed at 40°C protects cells against H_2O_2-induced cytotoxicity and apoptosis

The pretreatment of HeLa cells at a mild temperature of 40°C for 3h led to the development of thermotolerance, which was seen as subsequent resistance to toxicity of the pro-oxidant H_2O_2 (25-50 μM) (Figure 1). The loss of clonogenic cell survival (Fig. 1A) and induction of apoptosis (Fig. 1B-F) by H_2O_2 were diminished in thermotolerant cells compared to controls (maintained at 37 °C for 3 h). Apoptosis was evaluated by the condensation of nuclear chromatin (Fig. 1B and C), activation of effector caspase-3 (Fig. 1D) and cleavage of ICAD (Figs. 1E and F), which are late-stage events occurring in the execution phase. Very few (<5%) necrotic cells were seen under these conditions (Fig. 1B).

H_2O_2 induces up-regulation of the Fas death ligand (FasL): protective effect of mild thermotolerance

Subsequently, the ability of mild thermotolerance (40 °C) to protect cells against H_2O_2-induced apoptosis was investigated. The role of H_2O_2 in the activation of the mitochondrial pathway of apoptosis is well-established [Chandra et al, 2000], whereas activation of death receptor-mediated apoptosis by H_2O_2 is not completely understood

[Circu and Aw, 2010]. Apoptosis involving the death receptor Fas is activated by the binding of FasL to its receptor. To determine the role of H_2O_2 in the activation of death receptor signalling, we evaluated the extracellular expression of FasL in both thermotolerant and non-thermotolerant HeLa cells by flow cytometry. Exposure of cells to 5-25 μM H_2O_2 for 1 h caused an increase in FasL expression by 31-48% (Figs. 2A and B). Next, to confirm the role of H_2O_2 in the induction of FasL expression, cells were treated with the cell-permeative H_2O_2 scavenger, polyethylene glycol (PEG)-catalase. The induction of FasL expression by 5 μM H_2O_2 was completely inhibited by PEG-catalase (Figs. 2B and C). Treatment of HeLa cells with PEG-catalase (300 U/ml, 3 h) increased catalase activity by 46.1 ± 11.6%, compared to untreated controls. Interestingly, mild thermotolerance completely inhibited the peroxide-induced increase in FasL expression (Figs. 2A and B), which is an early event in the death receptor pathway.

Protective effect of mild thermotolerance against H_2O_2-induced FADD translocation to the plasma membrane

The ligation of FasL to its receptor Fas changes the conformation of the receptor and results in clustering as well as trimerization of the receptor, which is required for formation of the death-inducing signalling complex (DISC) at the plasma membrane. The components of the DISC include oligomerized Fas, the adaptor protein Fas-associated death domain (FADD) and procaspase-8. Activation of Fas triggers translocation of FADD from the cytosol to the plasma membrane. When HeLa cells treated with H_2O_2 for 1h, there was an increase in the expression of microsomal FADD (Figs. 3A and B), along with a corresponding decrease in cytosolic levels of FADD (Figs. 3C and D). In thermotolerant cells, the H_2O_2–induced increase in microsomal FADD expression was significantly reduced (Figs. 3A and B) and cytosolic levels of FADD were higher (Figs. 3C and D), compared to normal (non-thermotolerant) cells. The purity of microsomal and cytosolic fractions was 92 and 98%, respectively (Fig. 3E).

Caspase-8 and caspase-2 play important roles in H_2O_2-induced apoptosis: protective effect of mild thermotolerance

Auto-proteolytic cleavage of procaspase-8 at the DISC results in activation of initiator caspase-8. Exposure of HeLa cells to H_2O_2 (0-50μM) for 1 h caused procaspase-8 (55 kDa) cleavage and a marked increase in levels of the 20 kDa cleavage fragment (Figs. 4A and B). The activation of caspase-8 by H_2O_2 was confirmed by an enzymatic assay (Fig. 4C). Caspase-8 activation was completely inhibited by PEG-catalase (Fig. 4D), confirming the role of H_2O_2 in caspase-8 activation. Mild thermotolerance (40°C) markedly decreased levels of the caspase-8 cleavage fragment and caspase-8 activation, compared to control cells (Figs. 4A, B, and C). Recent studies showed that recruitment of caspase-8 to the DISC can also lead to activation of caspase-2 [Olsson et al, 2009; Manzl et al, 2009]. H_2O_2 also caused enzymatic activation of caspase-2 in HeLa cells (Fig. 5A). Accordingly, H_2O_2-induced caspase-2 activation was decreased partially by a caspase-8 inhibitor, Z-IETD-FMK (Fig. 5B). Caspase-2 activation by H_2O_2 was inhibited in thermotolerant cells (Fig. 5A).

The roles of caspase-8 and caspase-2 in apoptosis induced by H_2O_2 were investigated using inhibitors of caspase-8 and caspase-2. Indeed, the induction of nuclear chromatin condensation by H_2O_2 was decreased partially by inhibitors of caspase-8 (Figs. 4E and F) and caspase-2, (Figs. 5C and D), Z-IETD-FMK and Z-VDVAD-FMK, respectively.

H_2O_2 activates the cross-talk pathway mediated by Bid cleavage: inhibition by mild thermotolerance

Once activated, caspase-8 and caspase-2 can induce downstream events of apoptosis by two distinct mechanisms. One is by direct cleavage of effector caspase-3 and the second is by means of cross-talk between the death receptor and mitochondrial pathways, through cleavage of the pro-apoptotic protein Bid. The cleaved fragment t-Bid can then translocate to mitochondria and activate mitochondrial apoptosis. Exposure of HeLa cells

to H_2O_2 (0-50 μM) for 1 h caused cleavage of Bid and translocation of t-Bid to mitochondria (Fig. 6). Cytosolic levels of Bid decreased (Figs. 6A and B) while levels of t-Bid increased in the mitochondrial fraction (Figs. 6C and D). The purity of the mitochondrial fraction was 97% (Fig. 3E). Thermotolerance developed at 40°C afforded protection against H_2O_2-induced Bid cleavage and t-Bid translocation (Fig. 6). The inhibition of Bid cleavage (Figs. 7A and B) and mitochondrial t-Bid translocation (Figs. 7C and D) by inhibitors of caspase-8 and caspase-2 confirmed the role of these two caspases in H_2O_2-induced Bid cleavage.

Translocation of t-Bid to mitochondria leads to activation of the mitochondrial pathway of apoptosis involving initiator caspase-9 [Strasser et al, 2009]. Indeed, H_2O_2 (0-50 μM) caused caspase-9 activation, which was diminished partially by inhibitors of caspase-8 and caspase-2 (Fig. 8A). Furthermore, H_2O_2-induced activation of effector caspase-3 was also inhibited by the caspase-8 inhibitor (Fig. 8B).

Role of the Fas in H_2O_2-mediated death receptor apoptosis

To further confirm the role of FasL in apoptosis induced by H_2O_2, we determined whether inhibition of Fas activation could block H_2O_2-induced apoptosis. Kp7-6 is an irreversible inhibitor of Fas activation. It acts by binding with both the Fas and FasL, therefore preventing FasL from forming a stable complex with Fas [Hasegawa et al, 2004]. We therefore evaluated whether Kp7-6 could inhibit H_2O_2-induced apoptosis. Pre-treatment of cells with Kp7-6 effectively inhibited H_2O_2-induced nuclear chromatin condensation by about 50% (Figs. 9A and 9B). Moreover, Kp7-6 inhibited H_2O_2-induced activation of caspase-8 (Fig. 10A), caspase-2 (Fig. 10B) and caspase-9 (Fig. 10C). Together, these findings show that H_2O_2 can activate apoptosis through the Fas death receptor in HeLa cells. Furthermore, the mitochondrial pathway of apoptosis was activated, at least in part, by the death receptor pathway through the cross-talk pathway.

Role of p53 as an upstream factor in activation of the death receptor pathway by H_2O_2

The tumor suppressor protein p53 can be activated by various DNA damaging agents [Yoshida and Miki, 2010], including the pro-oxidant H_2O_2 [Pallepati and Averill-Bates, 2010; Liu et al, 2008]. At the transcriptional level, activated p53 can induce the expression of pro-apoptotic proteins such as Bax, FasL, Puma and Noxa, and suppress anti-apoptotic proteins such as Bcl-2 [Yoshida and Miki, 2010]. To determine whether induction of FasL expression by H_2O_2 requires upstream p53 activation, cells were pre-treated with a p53 inhibitor, pifithrin-α. Indeed, pifithrin-α inhibited the induction of FasL expression by H_2O_2 (10-25 μM) (Figs. 10D and E). Furthermore, H_2O_2-induced caspase-8 activation was markedly reduced by pifithrin-α (Fig. 10A). The activation of caspase-2 by H_2O_2 was decreased partially by pifithrin-α (Fig. 10B). These results suggest that p53 is an upstream factor in H_2O_2-mediated activation of death receptor signalling, leading to induction of FasL and caspase-8 activation, which, in turn, activates initiator caspase-2. However, activation of caspase-9 in the mitochondrial pathway was not inhibited by pifithrin-α (Fig 10C). This shows that H_2O_2 can activate the mitochondrial pathway directly, or by alternate mechanisms that are independent of p53 activation.

The activation of execution phase events of apoptosis by H_2O_2 was also dependent on p53. Caspase-3 activation (Fig. 11A) and nuclear chromatin condensation (Figs. 11B and C) were inhibited partially by pifithrin-α.

DISCUSSION

The findings of this study contribute to knowledge about adaptive responses, in that mild thermotolerance developed at 40°C protects cells against H_2O_2-induced apoptosis through the death receptor signalling pathway. Moreover, they improve understanding

about activation of death receptor-mediated apoptosis by H_2O_2 in HeLa cells. Activation of the death receptor pathway, upon exposure to 5-50 μM H_2O_2 for 1h to 3h, was evident by increased expression of FasL and recruitment of FADD and procaspase-8 to the plasma membrane. Once activated, caspase-8 processed caspase-2, which is a novel finding in this study. The conditions (25-50 μM H_2O_2) that induced apoptosis also decreased clonogenic cell survival, which represents loss of cell proliferation.

Role of caspase-2 in H_2O_2-induced apoptosis

This study is the first to show the role of caspase-2 in apoptosis induced by H_2O_2, where a caspase-2 inhibitor reduced pro-oxidant-induced Bid cleavage, mitochondrial tBid translocation and chromatin condensation. Despite the early discovery of caspase-2, its physiological role still remains a puzzle [Krumschnabel et al, 2009]. Caspase-2 seems to have multiple roles in processes such as apoptosis, DNA repair and tumor suppression. Activation of caspase-2 has been reported during apoptosis induced by anti-Fas, cytokine deprivation, β-amyloid, etoposide and other stress stimuli [Zhivotovsky and Orrenius, 2005]. Caspase-2 can be activated by the PIDDosome in response to DNA damage [Maiuri et al, 2007; Tinel et al, 2007]. However, recruitment of caspase-8 to the DISC can lead to caspase-2 activation in the absence of PIDDosome formation [Olsson et al, 2009; Manzl et al, 2009]. Thus, there exist different routes by which caspase-2 can be activated.

Activation of the cross-talk pathway during H_2O_2-induced apoptosis

The cross-talk pathway that connects the mitochondrial and death receptor pathways was activated by H_2O_2 in HeLa cells. Cleavage of Bid and mitochondrial t-bid translocation were completely suppressed by caspase-8 and -2 inhibitors. This suggests that both caspase-8 and caspase-2 can process Bid and contribute to mitochondrial amplification of cell death by apoptosis (Scheme 1). However, post-mitochondrial caspase-9 activation by H_2O_2 was only partially decreased by inhibitors of caspase-8 and

-2. This indicates that H_2O_2 can activate the mitochondrial pathway in part through the death receptor pathway, and by alternate mechanisms that are independent of death receptor activation. Indeed, direct activation of mitochondrial apoptosis by H_2O_2 has been reported in several cell types [Pallepati and Averill-Bates, 2010; Chandra et al, 2000; Singh and Singh, 2008].

Role of Fas in H_2O_2-induced death receptor apoptosis

The Fas signalling pathway plays a critical role in regulation of homeostasis in the immune system, and is also involved in stress-induced apoptosis [Strasser et al, 2009]. Our findings show that H_2O_2 can induce FasL expression (Scheme 1). The role of Fas in H_2O_2-induced death receptor apoptosis is confirmed by inhibition of caspase-8/-2 activation and chromatin condensation by the Fas/FasL antagonist Kp7-6.

In other studies, H_2O_2 induced apoptosis through death receptors, but often at higher concentrations and longer exposure times than those in HeLa cells. In murine intestinal epithelial cells, H_2O_2 (50-200 μM, 4-6h) increased mRNA levels for FasL and Fas after 18h [Denning et al, 2002]. H_2O_2 (200-1000 μM, 24h) caused up-regulation of Fas in HUVEC cells [Suhara et al, 1998]. A more rapid response occurred in HL-60 cells where 50 μM H_2O_2 (2h) activated caspase-8 and caspase-3 [Zhuang et al, 2000]. However, H_2O_2 (50-100 μM, 12h) induced apoptosis by a Fas-independent mechanism involving mitochondria in a human T cell line (CEM C7) [Dumont et al, 1999]. The mechanisms of H_2O_2-induced apoptosis are clearly dependent on cell type.

Role of p53 as an upstream factor in H_2O_2-induced death receptor apoptosis

This study shows that pifithrin-α decreased FasL up-regulation, activation of caspase-8 and caspase-2, and execution phase events, confirming that p53 is an upstream factor in death receptor activation during H_2O_2-induced apoptosis (Scheme 1). p53 also played a role in mitochondrial apoptosis by causing induction of Puma and translocation of Bax to

mitochondria [Pallepati and Averill-Bates, 2010]. Apoptosis was associated with increased p53 protein expression in several cell types including rat neural AF5 (800 μM H_2O_2, 24h), glioma (1 mM H_2O_2, 24h) and HeLa cells (50 μM H_2O_2, 1h) [Pallepati and Averill-Bates, 2010; Datta et al, 2002; McNeill-Blue et al, 2006].

Mild thermotolerance developed at 40°C protects HeLa cells against H_2O_2-induced death receptor apoptosis

The dose-response relationship is a key factor in determining whether a certain compound or stress causes an adverse effect. Low dose, chronic exposure to different stresses such as heat shock, ROS and radiation can induce adaptive responses, which allow cells and organisms to continue normal function in the face of a stimulus [Holsapple and Wallace, 2008]. Pre-exposure of cells to low doses of H_2O_2 or heat shock (45 min at 45°C) was shown to induce subsequent resistance to H_2O_2 [Spitz et al, 1987]. Adaptive responses often involve multiple changes in gene and protein expression, including the induction of cellular defenses (e.g. Hsps, antioxidants, etc.) to enable the cell to survive [Davies, 2000]. Hsp27 appears to be involved in mediating an adaptive response to ROS-generating agents such as carcinogens, anticancer drugs and other xenobiotics [Huot et al, 1996]. Mild thermotolerance developed at 40°C increased the expression of 6 Hsps (27, 32, 60, 70, 90 and 110 kDa) [Bettaieb and Averill-Bates, 2008]. The induction of antioxidants during development of thermotolerance could be an adaptive response resulting from increased ROS generation during mild heat shock at 40°C [Pallepati and Averill-Bates, 2010]. If an adaptive response cannot counteract an adverse stress exposure, then cells will be eliminated by death processes such as apoptosis or necrosis [Davies, 2000].

Our findings show that mild thermotolerance induced at 40°C created an apoptosis-resistant phenotype; activation of the death receptor signalling pathway (FasL up-regulation, caspase activation, Bid cleavage) was inhibited in HeLa cells treated with

H_2O_2. This apoptosis-resistant phenotype could be conferred by increased levels of both Hsps and antioxidants. Fas-mediated induction of apoptosis was regulated by Hsp70 and Hsp27 [Liossis et al, 1997; Mehlen et al, 1996; Schett et al, 1999; Zhao et al, 2007]. Hsp27 and Hsp70 regulated the death receptor pathway by preventing t-bid translocation to mitochondria, which in turn inhibits cytochrome c release [Gabai et al, 2002; Paul et al, 2002]. Hsp90-α was shown to be a negative regulator of caspase-2 activation [Bouchier-Hayes et al, 2009]. Furthermore, Hsp70 and phosphorylated Hsp27 can protect cells against oxidative stress. [Huot et al, 1996; Musch et al, 1996; Chen et al, 1999; Komatsuda et al, 1999]. Hsp27, Hsp70 and Hsp90 can attenuate apoptosis upstream of mitochondria [Steel et al, 2004], as well as interfering with apoptosome formation, post-mitochondrial events and caspase activation [Beere, 2004]. Thus, Hsps play a complex role in the regulation of apoptosis. Scheme 2 summarises the protective role of different Hsps against activation of death receptor-mediated apoptosis by H_2O_2.

CONCLUSION

This study advances knowledge about the protective effect of adaptive responses induced by exposure to mild stresses, such as fever temperatures, against ROS. Furthermore, new insights are provided about activation of the death receptor pathway of apoptosis by the pro-oxidant H_2O_2, which has important repercussions for human health, given the role of ROS in major physiopathologies such as cancer, diabetes, cardiovascular diseases and neurodegenerative disorders.

ACKNOWLEDGMENTS

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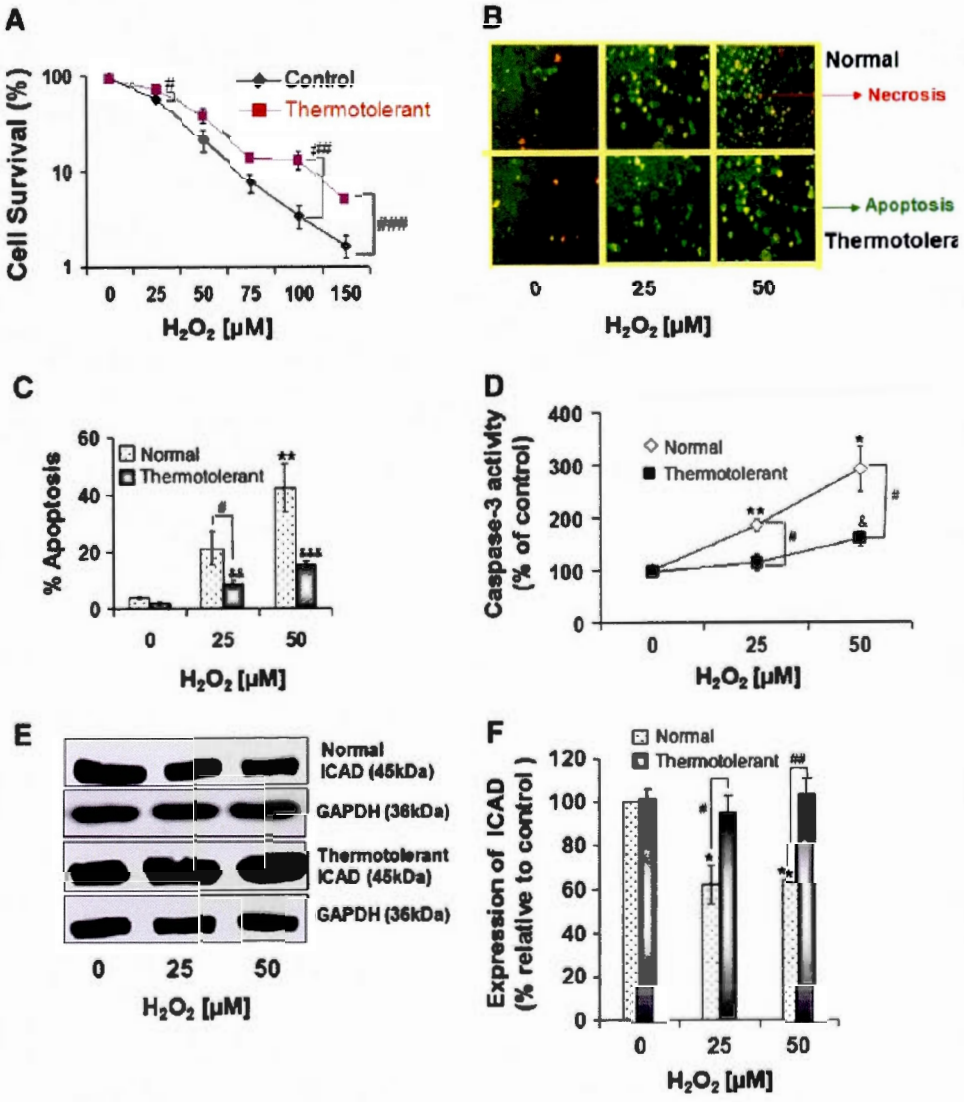


Figure 1 Protective effect of mild thermotolerance against H_2O_2-induced chromatin condensation and cytotoxicity. Thermotolerant (3h, 40°C) and nonthermotolerant HeLa cells (3 h, 37 °C), were treated with H_2O_2 (0-150μM) for (A) 2 h or (B-F) 3 h. (A) Cytotoxicity of H_2O_2 in thermotolerant versus normal cells was assessed by a clonogenic cell survival assay. The control value represents 10^5 cells and was normalized to 100% cell survival. For curve analysis of thermotolerant versus non-thermotolerant cells, p=0.0002. (B) Nuclear morphology was assessed by fluorescence microscopy using

Hoechst 33258 to detect blue-green apoptotic cells and propidium iodide to detect red necrotic cells (magnification 320 X). Images are representative of a minimum of 3 independent experiments. (C) The fractions of apoptotic cells are given relative to total cells. (D) Enzymatic activity of caspase-3 was measured in cell lysates using fluorogenic substrate (Ac-DEVD-AMC), and was expressed relative to the control at 37°C (100%). (E) Immunodetection of ICAD (45 kDa) in whole cell lysates was carried out by Western blotting, using GAPDH (36 kDa) as loading control. A representative blot is shown from three independent experiments. (F) ICAD protein expression from densitometric analyses is relative to the control at 37°C. (A, C, D, F) Data represent means and SEM from a minimum of three independent experiments. $P<0.05$ (*, &), $P<0.01$ (**, &&) or $P<0.001$ (***, &&&) indicates a statistically significant difference between treatment with H_2O_2 and the respective untreated control, for thermotolerant (&) or non-thermotolerant (*) cells. $P<0.05$ (#), $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± thermotolerance), for a given concentration of H_2O_2.

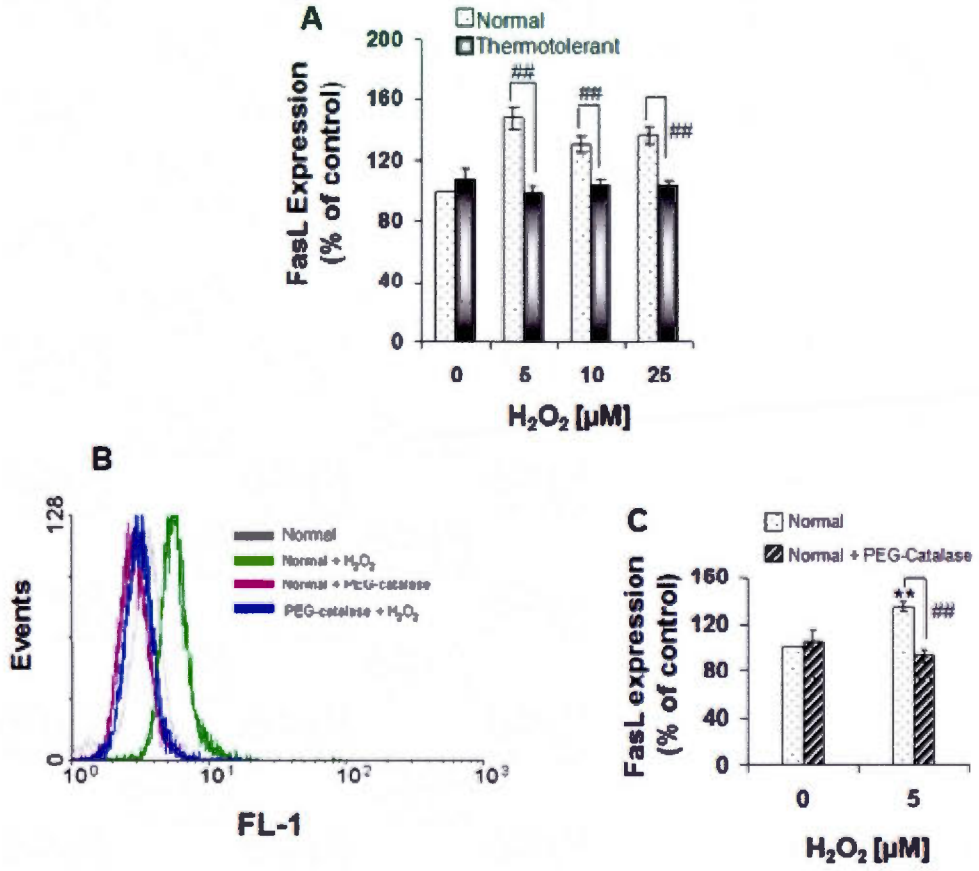


Figure 2 Mild thermotolerance protects cells against induction of Fas ligand expression by H_2O_2. (A) Thermotolerant (3 h, 40 °C) and nonthermotolerant HeLa cells were treated with H_2O_2 (0-25 μM) for 1 h. (B, C) Nonthermotolerant cells were pre-incubated for 3 h with or without PEG-catalase and then treated with H_2O_2 (0-5 μM) for 1 h. FasL expression was determined by flow cytometry and is shown relative to untreated controls (no H_2O_2) at 37°C, designated as 100%. Data represents means ± SEM from five independent experiments. (B) The histogram plots are representative results of FasL expression in control cells (no H_2O_2, grey line), and cells treated with 5μM H_2O_2 (green line), with PEG-catalase (no H_2O_2, pink line), or with PEG-catalase and H_2O_2 (blue line). (A, C) $P<0.01$ (**) indicates a statistically significant difference between treatment with H_2O_2 and the control. Significant differences between PEG-treated, or thermotolerant cells, and untreated controls (37°C), for a given concentration of H_2O_2, are represented by $P < 0.01$ (##).

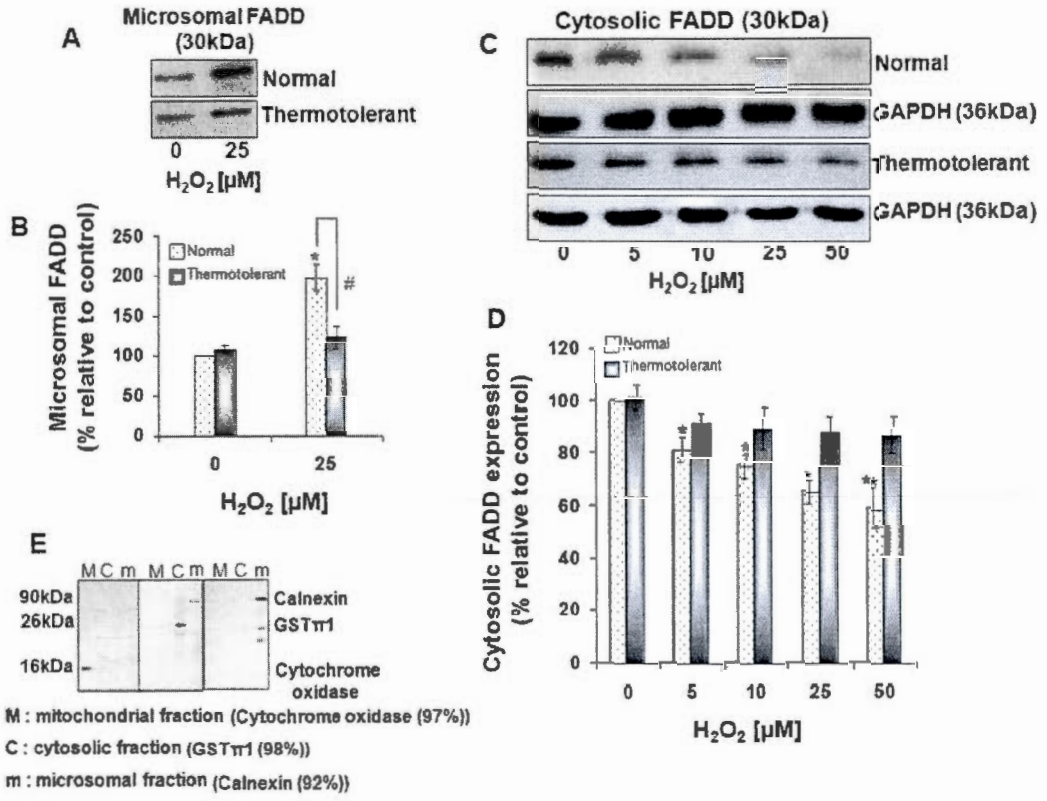


Figure 3 H_2O_2 causes translocation of the adaptor protein FADD to the plasma membrane: abrogation by mild thermotolerance developed at 40 °C. After H_2O_2 (0-50 μM) treatment for 1 h, FADD protein was immunodetected in thermotolerant (3 h, 40 °C) and nonthermotolerant (3 h, 37 °C) HeLa cells. A representative blot from three independent experiments is shown for FADD (30 kDa) expression (A) in microsomal and (C) cytosolic fractions, using GAPDH (36 kDa) as loading control for the cytosolic fraction. Densitometric analyses for expression of (B) microsomal and (D) cytosolic FADD are relative to non-thermotolerant controls at 37°C (100%). (E) A representative blot is shown for purity of subcellular fractions, using calnexin, glutathione S-transferase (GST-π1) and cytochrome oxidase for microsomal (m), cytosolic (C) and mitochondrial (M) fractions, respectively. $P < 0.05$ (*) or $P < 0.01$ (**) indicates a statistically significant difference between H_2O_2 treatment and the control. $P<0.05$ (#) indicates a significant difference between treatments (± thermotolerance), for 25 μM H_2O_2.

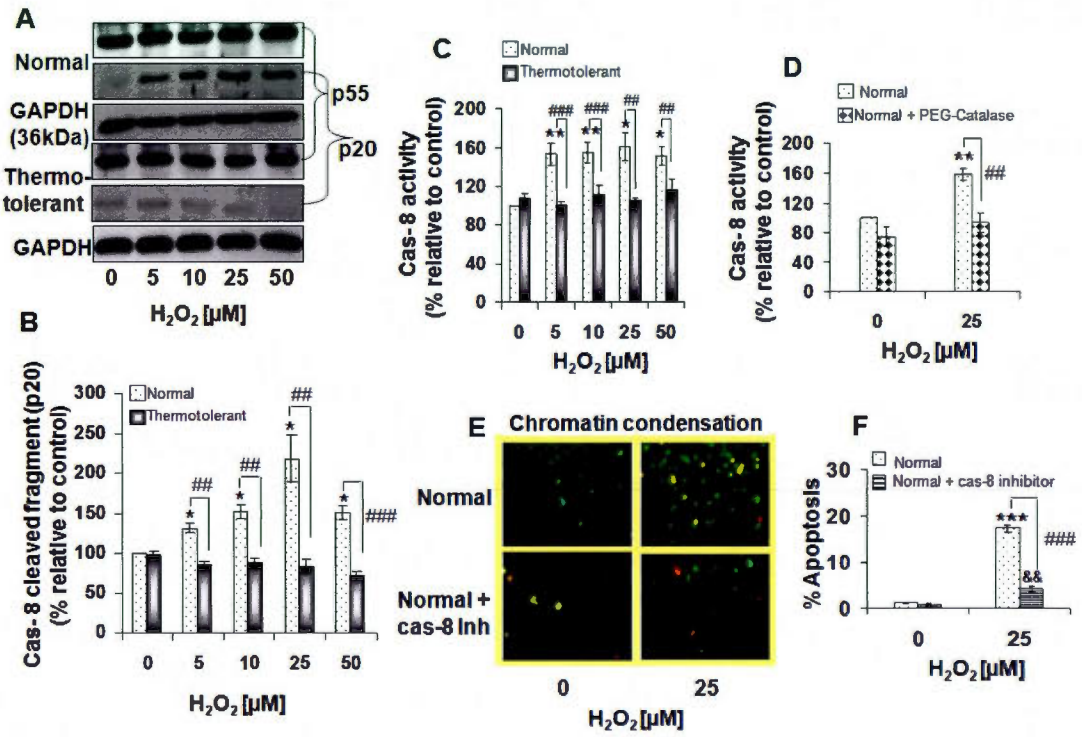


Figure 4 Caspase-8 plays a key role in H_2O_2-induced apoptosis: abrogation by mild thermotolerance. Thermotolerant cells (3 h, 40 °C) and nonthermotolerant (3 h, 37 °C) cells (pretreated with PEG-catalase, caspase-8 inhibitor, or no inhibitor), were incubated with H_2O_2 (0-50 μM) at 37 °C for 1 h (caspase-8) or 3 h (chromatin condensation). (A) Representative Immunoblots are shown from five independent experiments for levels of procaspase-8 (p55) and the p20 cleavage fragment. (B) Protein levels were analyzed by densitometry and expressed relative to non-thermotolerant cells at 37 °C (100%). (C, D) Activity of caspase-8 (1h) in (C) thermotolerant and nonthermotolerant cells, or (D) normal cells with or without PEG-catalase, were expressed relative to untreated controls (no H_2O_2) at 37°C (100%). (E) Chromatin condensation was visualized using Hoechst 33258 (magnification 320×). (F) The fraction of apoptotic cells following treatment with H_2O_2 for 3 h is given relative to total cells. $P < 0.05$ (*), $P < 0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between treatment with H_2O_2 and the control (&& is relative to control for caspase-8 inhibitor). $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± thermotolerance, ± inhibitors), for a given concentration of H_2O_2.

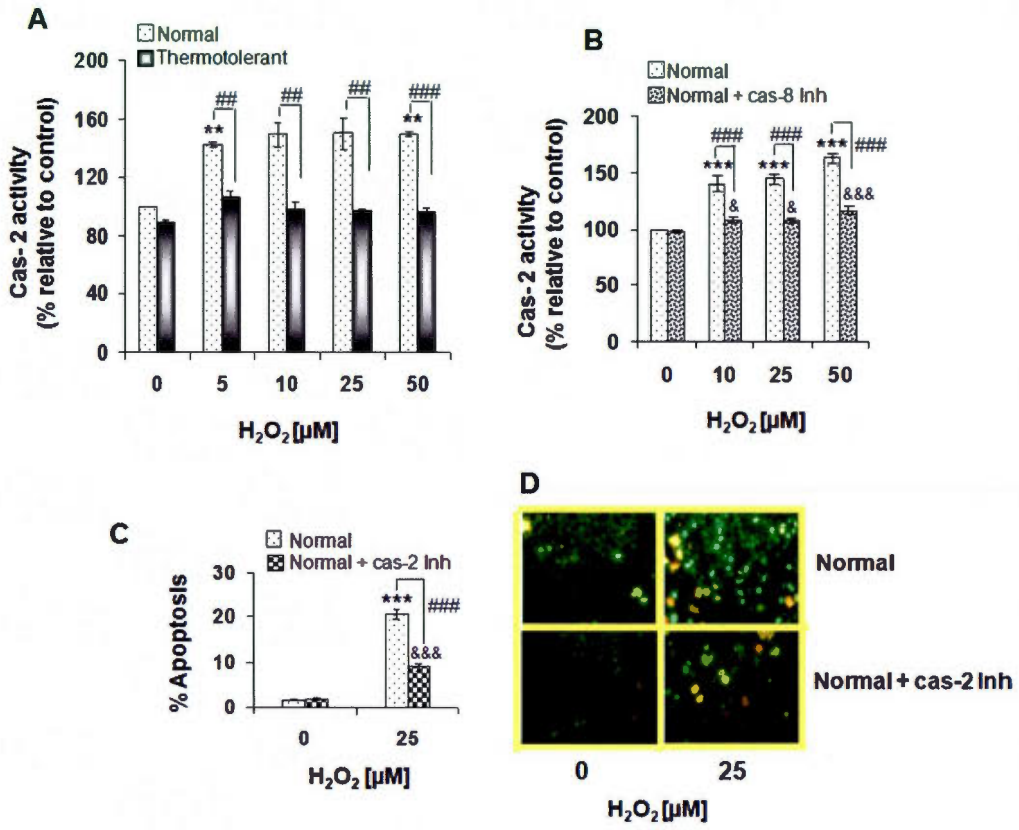


Figure 5 Mild thermotolerance (40°C) inhibits H_2O_2-induced caspase-2 activation. Thermotolerant (3 h, 40 °C) and nonthermotolerant (3 h, 37 °C) HeLa cells were incubated at 37 °C with H_2O_2 (0-50 µM) in D-MEM containing 10% FBS. (A, B) Caspase-2 activity (1 h) was measured in cell lysates using the fluorescent substrate (Z-VDVAD-AFC). Activity of caspase-2 in (A) thermotolerant and non-thermotolerant cells, or (B) normal cells with or without caspase-8 inhibitor (B), was expressed relative to controls at 37°C (100%). Data represent mean ± SEM from seven independent experiments. (C, D) Cells were pretreated with a caspase-2 inhibitor (Z-VDVAD-FMK, 25 µmol/L, 1 h) before exposure to 25 µM H_2O_2 for 3 h. (C) Cells were stained with Hoechst and PI and visualized by fluorescence microscopy (magnification 320 X). (D) The fraction of apoptotic cells following treatment is given relative to total cells. $P < 0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between treatment with H_2O_2 and the untreated control (for & and &&&, relative to inhibitor control). $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± thermotolerance, ± caspase-8 or -2 inhibitor), for a given concentration of H_2O_2.

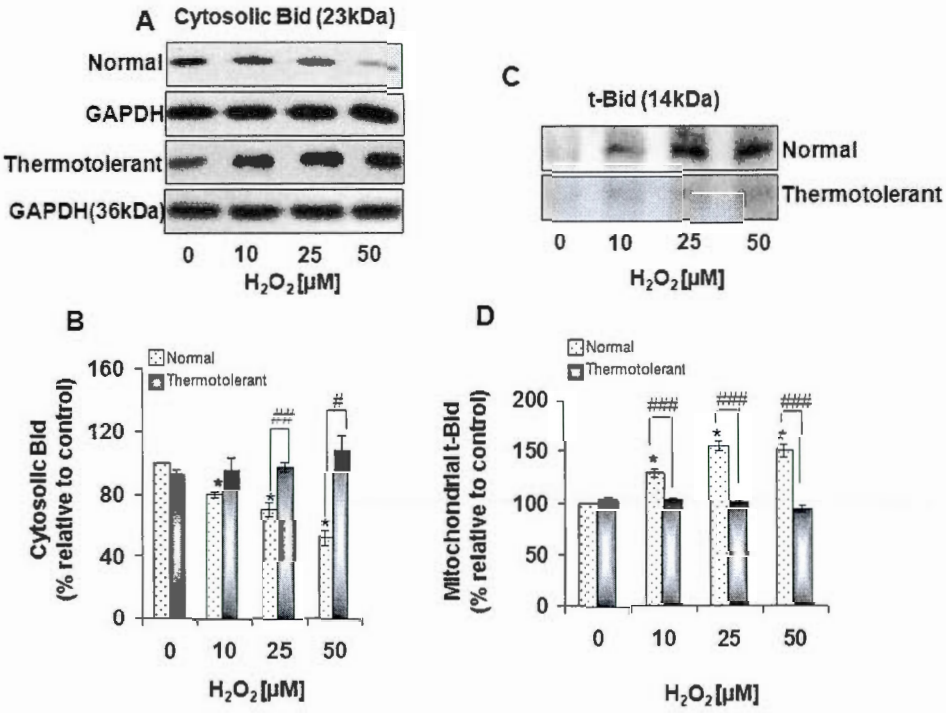


Figure 6 H_2O_2-acivates the Bid-mediated cross-talk pathway: protection by thermotolerance at 40 °C. Thermotolerant (3 h, 40 °C) and nonthermotolerant (3 h, 37°C) HeLa cells were incubated for 1 h at 37 °C with H_2O_2 (0-50 μM). Representative Immunoblots from five individual experiments are shown for the expression of Bid (23 kDa) in cytosolic fractions (A) and t-Bid (14 kDa) in mitochondrial fractions (C), using GAPDH as loading control for cytosolic fractions. (B, D) Densitometric analyses are shown for Bid (B) and t-Bid (D) protein expression in thermotolerant and non-thermotolerant cells, relative to control cells at 37°C (100 %). $P<0.05$ (*) indicates a statistically significant difference between H_2O_2 treatment and the control. $P<0.05$ (#), $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± thermotolerance), for a given concentration of H_2O_2.

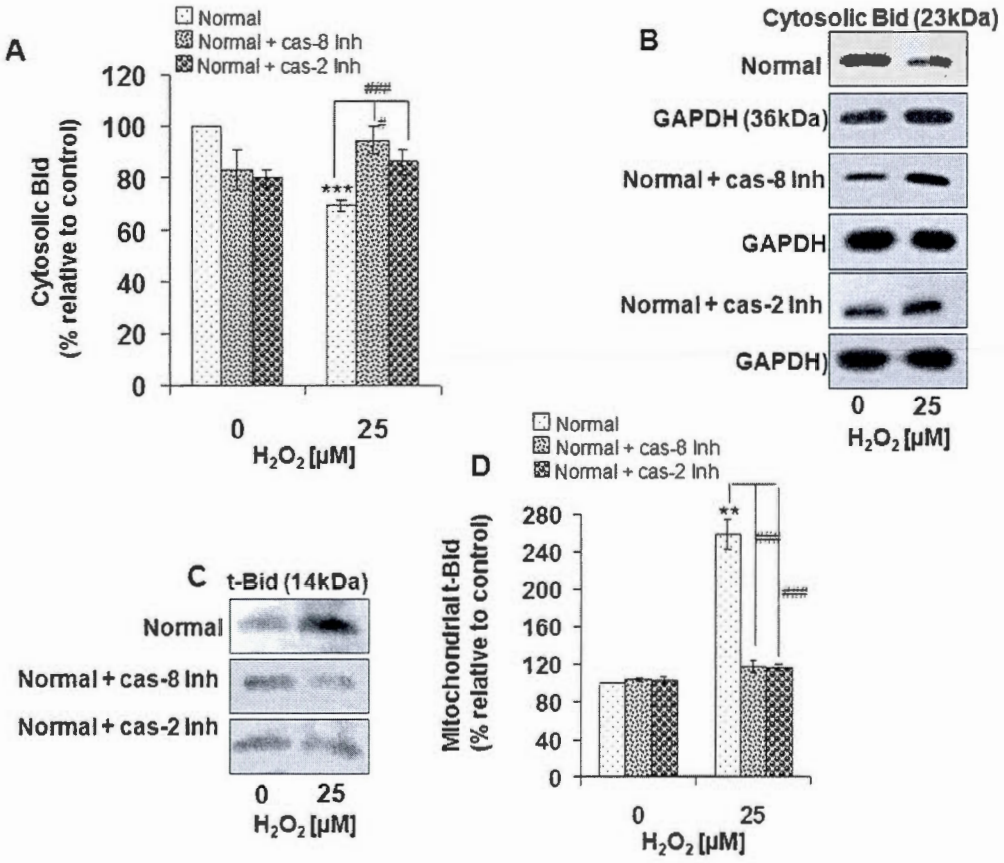


Figure 7 Roles of caspase-8 and caspase-2 in H_2O_2-mediated cleavage of Bid. HeLa cells (nonthermotolerant) were pretreated with inhibitors of caspase-8 or caspase-2, or no inhibitor, and then incubated with H_2O_2 (0, 25μM) for 1 h. Densitometric analyses of expression of (A) cytosolic Bid and (D) mitochondrial t-Bid are relative to control cells at 37°C (100%). Representative immunoblots from three individual experiments are shown for (B) cytosolic Bid (23 kDa) and (C) t-Bid (14 kDa). Purity of cytosolic fractions was determined using glutathione S-transferase (GST-π1) and mitochondrial fractions using cytochrome c oxidase (see Fig. 3E). $P<0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between H_2O_2 treatment and the control. $P<0.05$ (#) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± caspase inhibitors) for 25 μM H_2O_2.

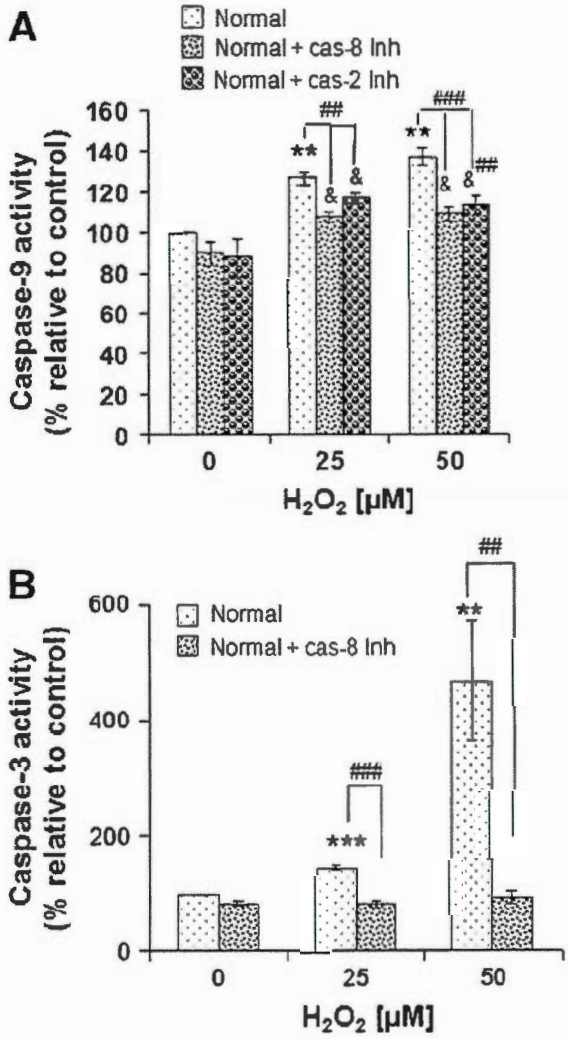


Figure 8 Caspase-9 activation by H_2O_2 is mediated by the cross-talk pathway. HeLa cells (nonthermotolerant) were pretreated with inhibitors of caspase-8 or caspase-2, or no inhibitor, and then incubated with H_2O_2 (0-50 μM) for 3 h. Enzymatic activities of (A) caspase-9 or (B) caspase-3, were detected using the fluorescent substrates Ac-LEHD-AFC or Ac-DEVD-AMC, respectively, and expressed relative to control cells (100%). Data represent mean and SEM from three independent experiments performed with multiple estimations per point. $P < 0.05$ (*), $P < 0.01$ (**) or $P<0.001$(***) indicates a statistically significant difference between treatment with H_2O_2 and the control (for &, relative to respective inhibitor control). $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± caspase inhibitor) for a given concentration of H_2O_2.

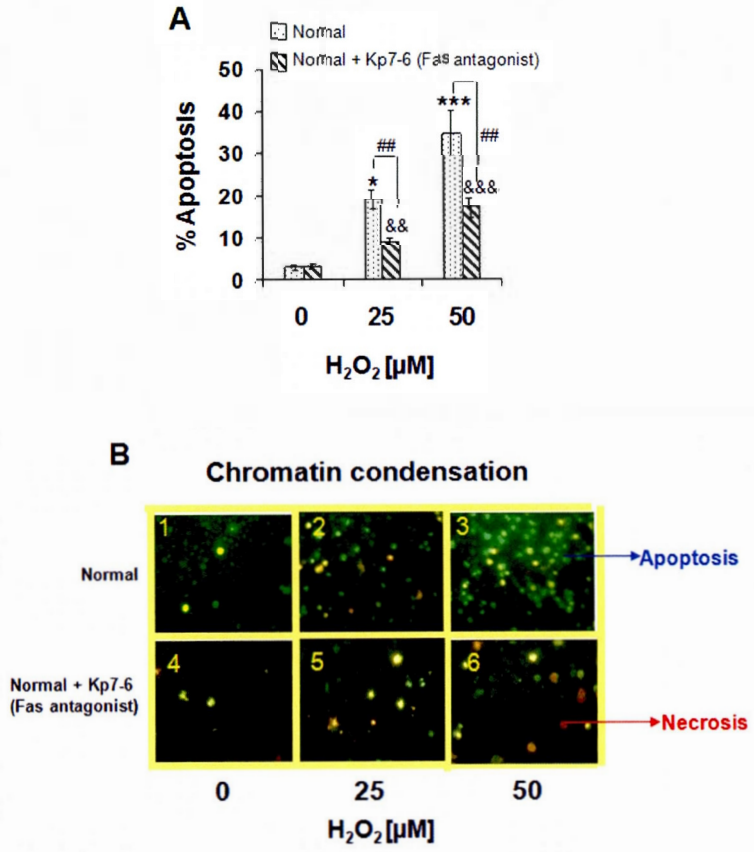


Figure 9 Role of the Fas in apoptosis mediated by H_2O_2. HeLa cells (non-thermotolerant) were preincubated with (B4-B6) or without (B1-B3) 1mM Kp7-6 (Fas/FasL antagonist) for 1 h and then incubated with H_2O_2 (0-50 µM) for 3 h. Cells were stained with Hoechst 33258 and PI (magnification 320 X). (A) The fraction of apoptotic cells (blue-green fluorescence) is given relative to total cells. Data represent means and SEM from four independent experiments. Significant differences between H_2O_2-treated cells and controls are represented by $P < 0.05$ (*) and $P < 0.001$ (***) (for &, relative to Kp7-6 control). $P<0.01$ (##) indicates a statistically significant difference between treatments (± Kp7-6) for a given concentration of H_2O_2.

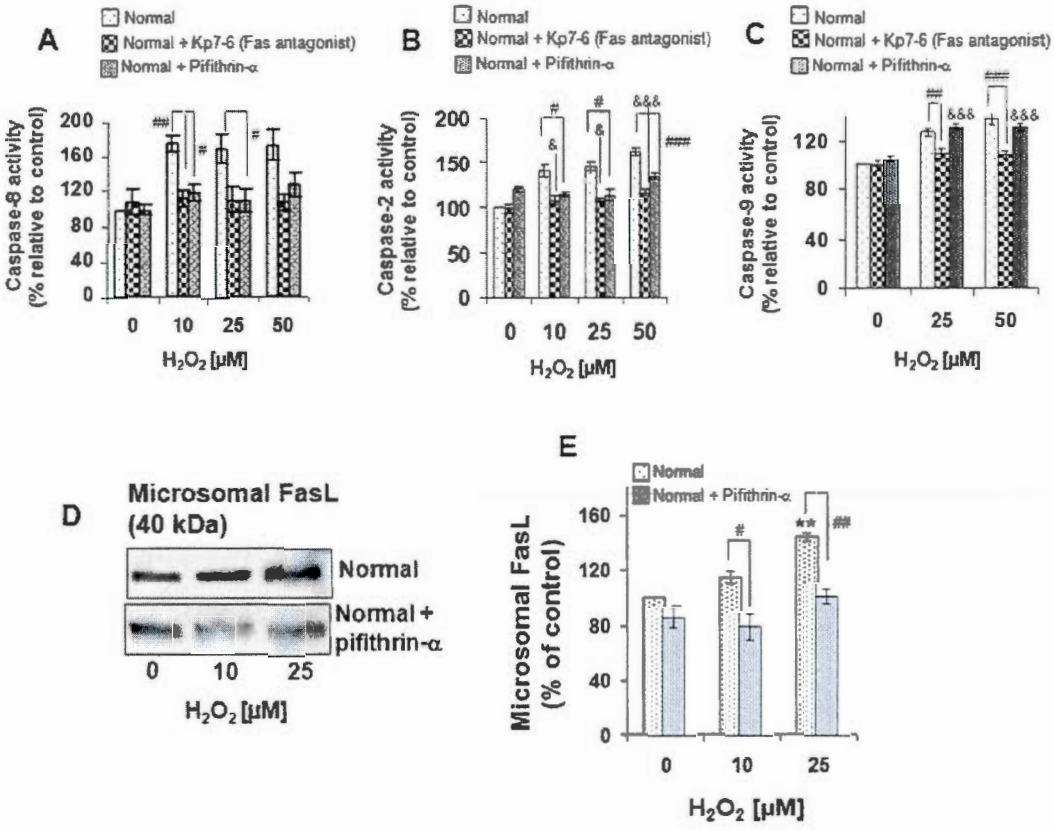


Figure 10 Role of p53 as an upstream factor in H_2O_2-mediated death receptor apoptosis. HeLa cells (nonthermotolerant) were pretreated with pifithrin-α or Kp7-6 or no inhibitor for 1 h and then incubated with H_2O_2 (0-50 μM) for 1 h (A, B, D, E) or 3 h (C). (A-C) Activities of caspase-8 (A), caspase-2 (B) and caspase-9 (C) in cells, with or without Kp7-6 or pifithrin-α, were expressed relative to untreated controls (100%). (D) A representative blot from three independent experiments for FasL protein expression in microsomal fractions is shown. (E) Densitometric analysis of FasL expression is shown relative to untreated controls (100%). $P<0.01$ (**) indicates a statistically significant difference between treatment with H_2O_2 and the control (for &, relative to inhibitor control). $P < 0.05$ (#), $P<0.01$ (##) or $P<0.001$ (###) indicates a statistically significant difference between treatments (± pifithrin-α, ± Kp7-6) for a given concentration of H_2O_2.

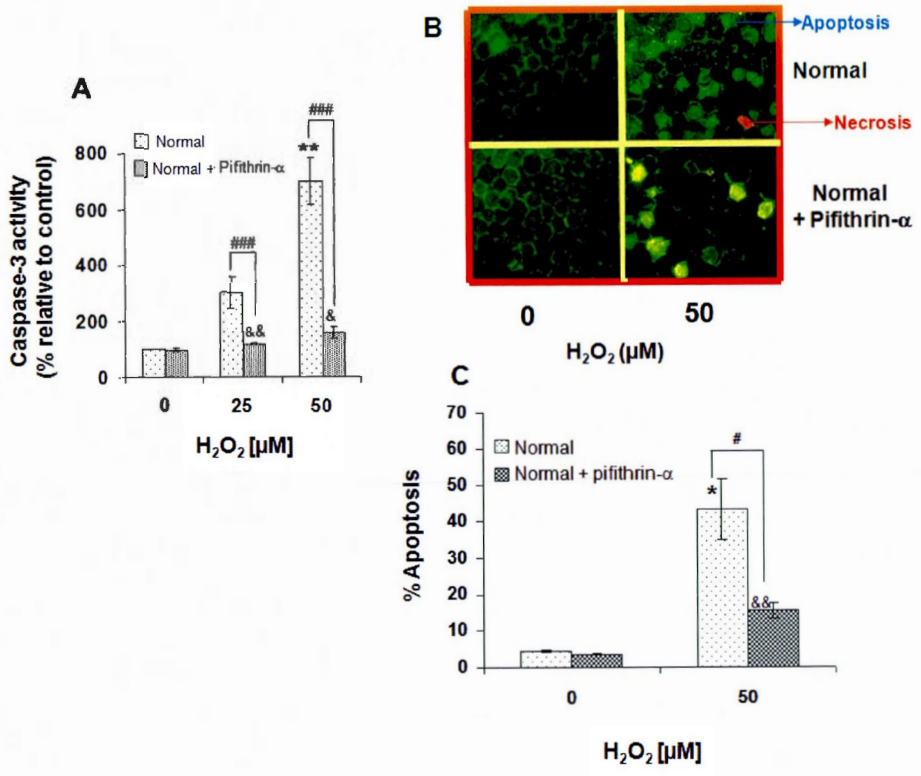
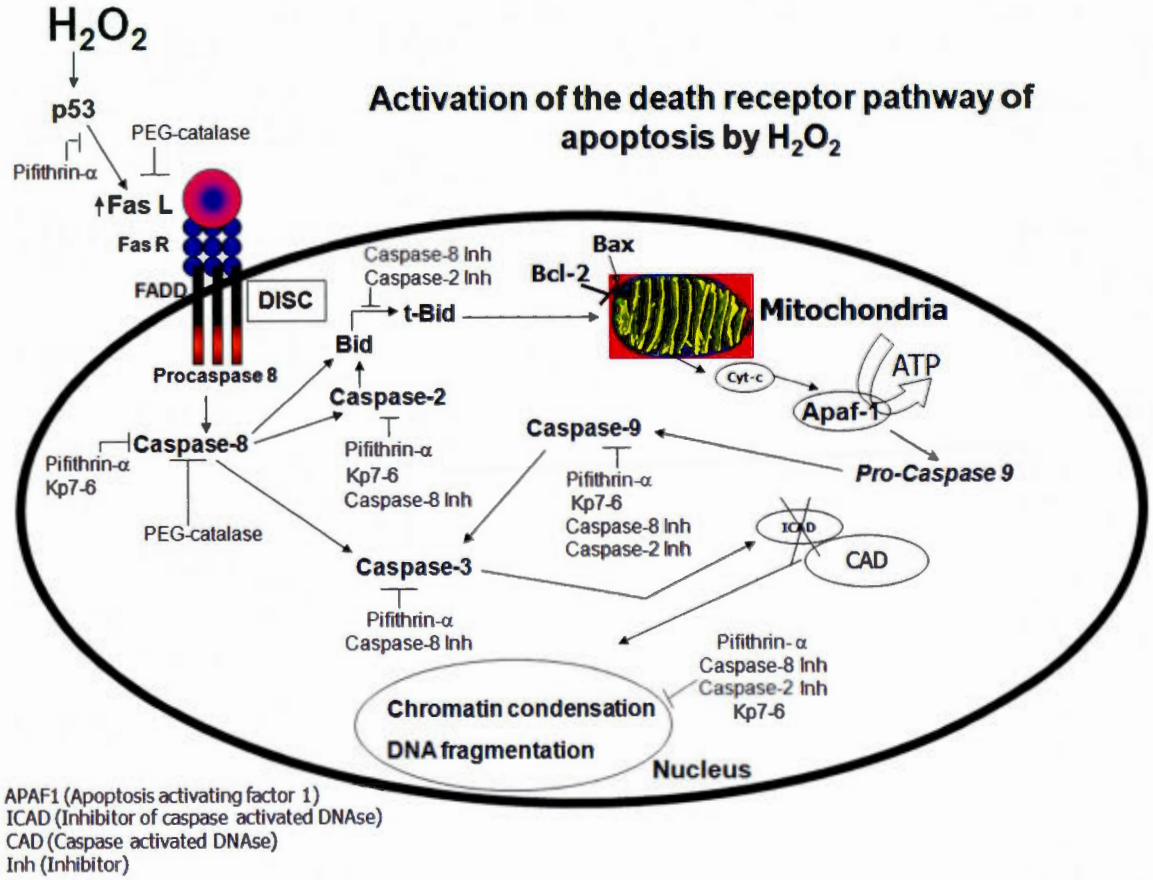
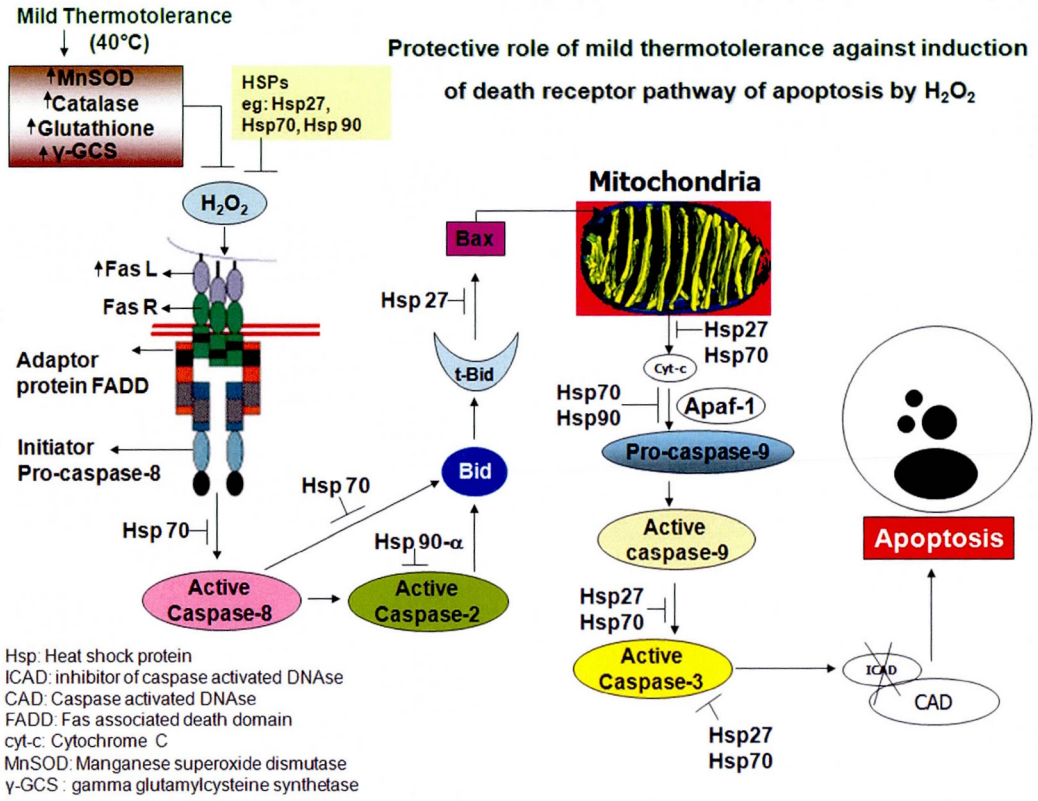


Figure 11 p53 inhibition decreases execution-phase events of H_2O_2-induced apoptosis. HeLa cells (nonthermotolerant) were pretreated with or without pifithrin-α for 1 h and then incubated with H_2O_2 (0-50 μM) for 3 h. (A) Enzymatic activity of caspase-3 was measured in cell lysates using Ac-DEVD-AMC, and was expressed relative to the control (100%). (B) Cells were stained with Hoechst and PI and visualized by fluorescence microscopy (magnification 320 X). Images are representative of a minimum of 3 independent experiments. (C) The fraction of apoptotic cells following treatment is given relative to total cells. Significant differences between H_2O_2-treated cells and untreated controls are represented by $P < 0.05$ (*) and $P < 0.01$ (**) (for &, relative to pifithrin-α control). $P<0.05$ (#) and $P<0.001$ (###) indicates a statistically significant difference between treatments (± pifithrin-α) for a given concentration of H_2O_2.



Scheme 1 Schematic representation of the p53-dependent death receptor pathway of apoptosis induced by hydrogen peroxide and mediated by Fas in HeLa cells.



Scheme 2 Summary of the protective role of mild thermotolerance developed at 40 °C in the regulation of the death receptor pathway of apoptosis induced by hydrogen peroxide.

2.4 ARTICLE III

ACTIVATION OF ER STRESS AND APOPTOSIS BY HYDROGEN PEROXIDE IN HELA CELLS: PROTECTIVE ROLE OF MILD HEAT PRECONDITIONING AT 40°C

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Note: References are included in the general reference section.

RÉSUMÉ

Le stress oxydatif peut interférer avec le repliement des protéines, ce qui peut conduire à une accumulation de protéines à la structure secondaire inexacte dans le réticulum endoplasmique (RE). Ce phénomène va activer la voie de réponse aux mauvais repliements de protéines (unfolded protein response, UPR) de façon à restaurer l'homéostasie du RE. Dans les cas où cette réponse adaptative ne parvient pas à empêcher les cellules de subir des dommages, celles-ci entrent en apoptose via leur RE. Le but de cette étude est d'examiner si la thermotolérance induite à une température modérée et non-létale (40°C) peut altérer l'induction par le peroxyde d'hydrogène (H_2O_2) de stress et d'apoptose liés au RE dans des cellules HeLa. L'expression des protéines PERK, p-PERK, eIF2α et p-eIF2α est augmentée dans des cellules thermotolérantes par comparaison avec des cellules non-thermotolérantes. Ce résultat constitue une preuve de l'augmentation des effets pro-survie de la voie PERK/eIF2α de l'UPR par une thermotolérance modérée. Une exposition courte (15 min) des cellules à un stress oxydatif (15-50 μM H_2O_2) donne lieu à l'activation de l'UPR, comme le confirment l'augmentation de l'expression de p-PERK, p-eIF2α, p-IRE1α et du clivage de ATF6. Une exposition plus longue (1-3h) au H_2O_2 induit l'apoptose liée au RE, durant laquelle l'expression de CHOP ainsi que l'activité enzymatique de la calpaïne et des caspase-7, -4, -12 et -3 augmentent. Tous ces évènements pro-apoptotiques et liés à la mort de cellules clonogéniques sont diminués dans les cellules thermotolérantes. L'activation des caspases -4 et -12 est diminuée par le chélateur de calcium BAPTA-AM ainsi que par des inhibiteurs de la calpaïne et de la caspase-7, ce qui confirme les rôles du calcium, de la calpaïne et de la caspase-7 dans l'activation par H_2O_2 de l'apoptose liée au RE. Dans les cellules thermotolérantes présentant un niveau de PERK diminué par siARN, la résistance à l'apoptose induite par H_2O_2 est partiellement inversée. Ceci prouve l'existence d'un lien de cause à effet entre le stress du RE et la résistance des cellules à l'apoptose induite par H_2O_2. Ces résultats montrent qu'une thermotolérance modérée joue un rôle protecteur et anti-apoptotique en augmentant le seuil d'induction de l'apoptose liée au RE par le stress oxydatif. De plus, la réponse adaptative (UPR) prédomine lors d'expositions au H_2O_2 courtes (stress modéré) tandis que lors d'expositions plus longues (stress sévère), c'est l'apoptose liée au RE qui est la plus fréquente. La compréhension des mécanismes moléculaires de la réponse adaptative à un environnement stressant ou extrême pose les bases de la compréhension des problèmes de santé liés à l'environnement et de l'identification de mécanismes de protection.

Mots clés: H_2O_2, apoptose, UPR, réticulum endoplasmique, caspase, CHOP.

ABSTRACT

The accumulation of misfolded proteins in the endoplasmic reticulum (ER) during stress conditions causes activation of the unfolded protein response (UPR). If this adaptive response cannot restore ER homeostasis, cells undergo ER-mediated apoptosis. This study determines whether thermotolerance developed at a mild temperature (40°C) can alter induction of ER-mediated stress and apoptosis by H_2O_2 in HeLa cells. Protein expression of PERK, p-PERK, eIF2α and p-eIF2α was increased in thermotolerant compared to non-thermotolerant cells. Thus, mild thermotolerance enhanced pro-survival effects of the PERK/eIF2α branch of the UPR. A short exposure (15 min) of cells to H_2O_2 (15-50 μM) activated the UPR: expression of p-PERK, p-eIF2α and p-IRE1α increased, and ATF6 cleavage occurred. Longer exposure (1-3h) to H_2O_2 induced ER-mediated apoptosis, whereby CHOP expression increased, and enzymatic activity of calpain, caspase-7, -4, -12 and -9 also increased. These pro-apoptotic events and clonogenic cell killing were all diminished in thermotolerant cells. Activation of caspases-4/-12 was decreased by the calcium chelator BAPTA-AM and by inhibitors of calpain and caspase-7, confirming the roles of calcium, calpain and caspase-7 in activation of ER-mediated apoptosis by H_2O_2. In thermotolerant cells with decreased levels of PERK by siRNA, there was partial reversal of resistance to H_2O_2-induced apoptosis. Hence, a causal connection exists between the ER stress response and resistance to H_2O_2-induced apoptosis. Mild thermotolerance plays a protective, anti-apoptotic role by increasing the threshold for induction of ER-mediated apoptosis by H_2O_2. Moreover, the adaptive response (UPR) dominates during milder H_2O_2 stress, whereas ER-mediated apoptosis occurs during more severe stress.

Keywords: H_2O_2, apoptosis, unfolded protein response, endoplasmic reticulum, caspase, calpain

INTRODUCTION

Reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (H_2O_2) are recognized as critical signaling molecules that take part in different cellular functions such as development, proliferation and differentiation [Circu and Aw, 2010]. ROS can trigger cellular dysfunction by damaging bio-molecules such as lipids, proteins, carbohydrates and nucleic acids [Ott et al, 2007]. Levels of ROS are tightly controlled by scavenging enzymes (e.g. catalase and superoxide dismutase (SOD)) and antioxidants (e.g. glutathione and vitamin E) that can detoxify them. When levels of pro-oxidants overwhelm the antioxidant defenses, disturbance of the redox equilibrium occurs, resulting in oxidative stress. This can lead to the activation of stress signaling pathways and transcription factors [Circu and Aw, 2010, Fulda et al, 2010]. Although the molecular mechanisms by which ROS activate these pathways are unclear, their activation can lead to different consequences, including growth arrest, senescence, up-regulation of death proteins, and cell death by apoptosis or necrosis.

Cells undergo apoptosis by three pathways involving death receptors, mitochondria and the endoplasmic reticulum (ER). Protein chaperones such as Bip/glucose-related protein 78 (GRP78), GRP94, calnexin and protein disulphide isomerase (PDI) assure correct folding of newly-synthesized proteins in the ER [Faitova et al, 2006]. A variety of conditions including glucose deprivation, hypoxia, disturbance of calcium homeostasis, and excess ROS can perturb ER function, leading to accumulation of unfolded proteins in the ER [Fulda et al, 2010; Schröder and Kaufman, 2005]. This phenomenon, known as ER stress, activates signaling pathways such as the unfolded protein response (UPR) and ER-associated protein degradation (ERAD). The UPR is a survival response that aims to recover normal cellular function in the face of adverse conditions. However, if UPR activation is not able to rescue cells from ER stress and correct the defects in protein folding, then cells are generally eliminated by ER-mediated

apoptosis [Fulda et al, 2010; Breckenridge et al, 2003; Malhotra and Kaufman, 2007; Rasheva and Domingos, 2009]. The ER stress response involves 3 distinct mechanisms: (i) translational attenuation to decrease the synthesis of new proteins; (ii) transcriptional activation of genes for ER chaperones and ERAD molecules; and (iii) ERAD, which involves translocation of misfolded or aggregated ER proteins to the cytoplasm, where they undergo proteosomal degradation [Schröder and Kaufman, 2005]. The activation of the UPR is mediated by three distinct ER stress sensors: protein kinase RNA (PKR)-like ER kinase (PERK), activating transcription factor-6 (ATF6) and inositol-requiring protein-1 (IRE1). In non-stressed cells, these sensors are retained in the ER lumen by interactions with Bip/GRP78. During ER stress, Bip releases these three sensors, leading to their activation [Schröder and Kaufman, 2005].

To counteract the multitude of processes that can trigger death, cells have developed a wide variety of survival strategies that can prevent inappropriate death [Portt et al, 2011]. The pre-exposure to sub-lethal doses of different stresses (e.g. ROS, heat shock, ischemia/reperfusion (IR)) can lead to adaptive responses that allow cells and organisms to continue normal function in the face of an adverse stimulus [Portt et al, 2011; Przybytkowski et al, 1986; Wiese et al, 1995; Kregel, 2002; Bettaieb and Averill-Bates, 2005; Holsapple and Wallace, 2008]. Adaptive responses appear to be mediated by a group of anti-apoptotic genes and their products (e.g. Hsps, antioxidants) that protect cells against diverse toxic and environmental stresses. If the adaptive response cannot protect the cell against an adverse exposure, then the damaged cell will be eliminated by apoptosis or necrosis [Portt et al, 2011; Holsapple and Wallace, 2008]. Thermotolerance is an adaptive survival response induced by heat preconditioning whereby cells become resistant to a subsequent lethal insult such as that triggered by heat shock, ROS or environmental stressors [Landry et al, 1982; Martindale and Holbrook, 2002]. This phenomenon is generally associated with the accumulation of heat shock proteins (Hsp) [Landry et al, 1982; Richter et al, 2010; Landry et al, 1989]. Thermotolerance can be

developed following shorter exposures (e.g. 30 min) to lethal temperatures (42-45°C) [15], or during continuous heating (e.g. 3-24 h) at non-lethal temperatures (39.5-41.5°C) [Przybytkowski et al, 1986]. The induction of thermotolerance by lower, fever-range temperatures such as 40°C has received little attention.

We recently reported that, besides Hsps [Bettaieb and Averill-Bates, 2005], mild thermotolerance (40°C) can induce several antioxidants in human cervical carcinoma (HeLa) cells [Pallepati and Averill-Bates, 2010]. The present work determines whether mild thermotolerance can induce other adaptive survival responses such as the UPR in HeLa cells. This study also investigates the role of the ER in H_2O_2-induced cell death, and whether mild thermotolerance (40°C) can alter the induction of ER stress by H_2O_2 and protect cells against ER-mediated apoptosis.

MATERIAL AND METHODS

Cell culture

HeLa cells (ATCC #CCL-2) were grown in monolayer in Dulbecco's modified Eagle's medium (Invitrogen Canada, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen Canada), in tissue culture flasks (Sarstedt, St. Laurent, QC, Canada) at 37 °C in a humidified atmosphere of 5% CO_2 in a water jacketed incubator [Bettaieb and Averill-Bates, 2008]. Culture medium was replaced with fresh medium 24 h before experiments. To induce thermotolerance, cells were transferred to an identical incubator for 3 h at 40 °C (± 0.1 °C), following a period of 20 min to allow the temperature of the culture medium to reach 40 °C [Przybytkowski et al, 1986]. The cells were grown to near confluence and then harvested using 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution, and washed by centrifugation (1000 x *g*, 3 min). There was no loss of viability in cells heated at 40 °C for 3 h, evaluated by trypan blue exclusion (data not shown).

Treatment with H_2O_2

Cells (10^6/ml) were incubated with H_2O_2 (Sigma-Aldrich, Canada, ON, Canada) (0, 15-50 μM) in DMEM containing 10% FBS at pH 7.4 for different times ranging from 15 min to 3 h at 37°C [Pallepati and Averill-Bates, 2010]. Cells were pretreated for 1 h with 50 μM calcium chelator BAPTA-AM (Sigma-Aldrich), 20 μM calpain inhibitor I (Ac-LLnL-CHO) (Sigma-Aldrich), 50 μM caspase-7/3 inhibitor I (5-[(S)-(+)-2-(methoxymethyl)pyrrolidino]sulfonylisatin) (Calbiochem), 20 μM caspase-4 inhibitor I, (LEVD-CHO) (Calbiochem) or 10 μM caspase-12 inhibitor (Z-ATAD- fluoromethyl ketone (FMK)) (MBL International Corporation), where indicated. Cells were washed by centrifugation (1000 x g, 3min) to remove inhibitors and H_2O_2, and then analyzed for the UPR response and ER apoptotic signaling.

Morphological analysis of apoptosis

Thermotolerant and non-thermotolerant cells (10^6/ml) were labeled with Hoechst 33258 (50 μg/ml) (blue-green fluorescence) (Sigma Chemical Co.), which binds to condensed nuclear chromatin of apoptotic cells [Tanel and Averill-Bates; 2005]. Cells were washed with PBS and then propidium iodide (PI) (50 μg/ml) was added to visualize necrotic cells (red fluorescence). Observations were made by fluorescence microscopy (model IM, Carl Zeiss Canada Ltd, St. Laurent, QC) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix imaging Inc, Mississauga, ON). Images were analyzed using Northern Eclipse software (Empix Imaging). Cells were classified using the following criteria: (1) Live cells with normal nuclei, pale blue/green chromatin with organized structure); (2) Apoptotic cells with bright blue /green condensed or fragmented chromatin); (3) Necrotic cells (red, enlarged nuclei with smooth normal structure). Fractions of apoptotic or necrotic cells were calculated relative to total cells. For each condition, 300 cells were counted.

Clonogenic cell survival assay

Clonogenic cell survival measures the ability of cells to undergo cell proliferation following a toxic insult. Non-thermotolerant cells and thermotolerant (40°C, 3h) cells were exposed to H_2O_2 (0-150 μM) for 2 h at 37°C in a final volume of 1.0 ml, in D-MEM containing 10% FBS. After the appropriate time, the cells were washed three times by centrifugation (1000 x g, 2 min) to stop the incubation [Pallepati and Averill-Bates, 2010]. The cells were resuspended in culture medium, diluted to the appropriate concentration and plated in tissue culture dishes (60 mm x 15 mm), which were incubated at 37°C incubator in an atmosphere of 5% CO_2 for 10 days. The dishes were then washed with PBS, fixed with 95% ethanol and stained with methylene blue before counting macroscopic colonies (>50 cells). Cytotoxicity was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells were seeded in the control plates, but where there was a loss of cell

survival, cells were plated at several different densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have previously demonstrated that, in this system, there is linearity between the number of cells plated and colonies formed over the range of $10\text{-}10^4$.

Preparation of whole cell lysates

For analysis of protein expression of PERK, p-PERK, eIF2α, p-eIF2α, ATF6, Bip, IRE1α and p-IRE1α, non-thermotolerant (37 °C, 3 h) and thermotolerant (40 °C, 3 h) cells were harvested and then exposed to H_2O_2 for 15 min. For detection of CHOP, calpastatin, caspase-4 and caspase-12, thermotolerant and non-thermotolerant cells were harvested and incubated with H_2O_2 at 37 °C for 1 or 2 h. Cells were washed by centrifugation (1000 x *g*, 3 min) in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.4) [Samali et al, 1999]. The supernatant was discarded, pelleted cells were resuspended in lysis buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 1 h. Then, the proteins of whole cell lysates were isolated in the supernatant, by a 10 min centrifugation step at 2500 x *g* to remove nuclei and unbroken cells [Bettaieb and Averill-Bates, 2008], and used for detection of proteins by Western blotting.

Preparation of subcellular fractions

Cells were treated with H_2O_2 and then subcellular fractions were prepared as described previously [Pallepati and Averill-Bates, 2010; Bettaieb and Averill-Bates, 2008]. Thermotolerant and non-thermotolerant cells were washed in buffer A and resuspended in buffer B containing 0.1 mM dithiothreitol (DTT). Lysates were homogenized using a dounce homogenizer (50 strokes/sample). After a 30 min incubation on ice, unbroken cells and nuclei were pelleted by centrifugation at 2500 x *g*

for 10 min. The supernatant was centrifuged further at 15,000 x *g* for 15 min to separate the mitochondrial fraction. The supernatant was further centrifuged at 100 000 x *g* for 1 h. The resulting supernatant (cytosolic fraction) as well as the pellet (resuspended in lysis buffer B and designated as the microsomal fraction), were used for protein detection of calpain.

Immunodetection

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins was carried out according to Laemmli (10%: PERK, p-PERK, IRE1α, p-IRE1α, Bip, calpain and calpastatin; 15%: eIF2α, p-eIF2α, ATF6, CHOP, caspase-4 and -12) [Laemmli, 1970]. Proteins (40 μg) were quantified according to Bradford [Bradford, 1976] and then solubilized in Laemmli sample buffer. Electrophoresis was carried out at a constant voltage of 125 V. Cellular proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Milli-pore, Bedford, MA, USA) [Tanel and Averill-Bates, 2005]. The membranes were probed with primary antibodies (see above proteins) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), in Tris-buffered saline (50 mM Tris base, 150 mM NaCl, 0.1% Tween-20) (TBS-T) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Biosource, Camarillo, CA, USA) diluted in TBS-T containing 5% milk powder. Proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA, USA). Protein expression was analyzed using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA), relative to GAPDH.

Determination of caspase or calpain activity by fluorescence spectroscopy

Cells were pretreated with or without calcium chelator BAPTA-AM, or inhibitors of calpain, caspase-7, caspase-4 and caspase-12, and then exposed to H_2O_2. After the

incubation, cells were washed, resuspended and then lysed at –80°C for 30 min. The kinetic reaction was followed for 30 min after addition of the appropriate caspase or calpain substrates at 37°C using a Quadruple Monochromator Microplate Reader (Infinite M1000, Tecan US, NC, USA). Caspase or calpain activities were measured by cleavage of the following fluorogenic peptide substrates: Suc-LY-AMC for calpain, Ac-DEVD-AMC for caspase-3, Ac-LEVD-AFC for caspase-4, MCA-Val-Asp-Gln-Val-Gly-Trp-Lys-(DNP)-NH_2 for caspase-7, Ac-ATAD-AFC for caspase-12 and Ac-LEHD-AFC for caspase-9 (Calbiochem) [Tanel and Averill-Bates, 2005]. Activities of caspases or calpain are represented as relative cumulative fluorescence of the kinetic reaction and compared to untreated controls.

Small interfering RNAs (siRNAs)

HeLa cells (0.25 x 10^6) were seeded in 6-well plates and allowed to reach 60-80% confluence on the day of transfection. The small interfering RNA (siRNA) constructs, PERK siRNA (sc-36213), the non-targeting siRNA control (sc-37007) and control siRNA-FITC conjugate A (sc-36869) were obtained from Santa Cruz. Cells were transfected for 48 h with 50 pmol of each siRNA construct, according to the manufacturer's protocol. Transfection efficiency (80%) was determined by counting FITC-positive cells by flow cytometry. Protein expression was determined in whole cell lysates (section 2.5) of transfected cells by 10% SDS-PAGE and Western blotting (section 2.7) using anti-PERK antibody (1:1000) (Santa Cruz). Transfected cells were made thermotolerant or non-thermotolerant and then analysed for H_2O_2-induced apoptosis.

Statistics

Data represent means ± SEM from at least 3 independent experiments performed in duplicate. When not shown, error bars lie within symbols. Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means.

The Bonferroni-Holmes adjustment was used to control for the Family-wise error rate at a desired level (α=5%). Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). For significant differences, $P < 0.05$.

RESULTS

Mild thermotolerance developed at 40°C protects HeLa cells against H_2O_2-induced apoptosis and clonogenic cell killing

Morphological analysis of nuclear chromatin condensation shows that exposure of cells to H_2O_2 (25 and 50 μM) for 3 h induced cell death by apoptosis (Figure 1Ab-1Ac, 1B), compared to the untreated control (Fig. 1a, 1B). Very few (<5%) necrotic cells were seen under these conditions. The treatment of HeLa cells at a mild temperature of 40°C for 3 h caused thermotolerance, which afforded significant protection against H_2O_2-induced apoptosis (Figure 1Ae-1Af, 1B). Thermotolerant cells were also resistant to H_2O_2-induced clonogenic cell killing (Figure 1C).

Mild thermotolerance (40°C) induces the UPR in HeLa cells

Mild thermotolerance developed at 40°C in HeLa cells induced several Hsps and antioxidants [Bettaieb and Averill-Bates, 2005; Pallepati and Averill-Bates, 2010]. These defense proteins could be involved in the protective effect of thermotolerance against H_2O_2-induced apoptosis [Pallepati and Averill-Bates, 2010]. In addition, the ability of mild thermotolerance (40°C) to evade H_2O_2-induced apoptosis might be associated with the activation/up-regulation of other survival responses such as ER stress. This study therefore determines whether mild thermotolerance (40°C) can induce the expression and/or activation of adaptive proteins of the UPR such as PERK, IRE1α and ATF6. Activation of PERK by oligomerization and autophosphorylation to its active form p-PERK, in turn phosphorylates eukaryotic initiation factor 2 (eIF2α), attenuating the translational process to reduce the load of newly synthesized proteins in the ER lumen

[Harding et al, 2000]. Indeed, the exposure of HeLa cells to mild heat (40°C) for 3 h caused significant activation (phosphorylation) of PERK (Fig. 2A, 2B) and eIF2α (Fig. 3A, 3B), compared to non-thermotolerant control cells. The levels of p-PERK and p-eIF2α were increased by 2.4- and 1.5-fold, respectively. In addition, protein expression of PERK (Fig. 2C, 2D) and eIF2α (Fig. 3C, 3D) increased by 1.4- and 1.2-fold, respectively. There was a small 1.2-fold increase in protein expression of the ER chaperone Bip, compared to the non-thermotolerant control (Fig. 4A, 4B). The levels of p-IRE1α, IRE1α (Fig. 7C), ATF6 and cleaved ATF6 (Fig. 8A, 8D) and were unchanged in mild thermotolerant cells. Together, these results show that mild thermotolerance enhanced the pro-survival effects of the PERK/eIF2α branch of the UPR.

Activation of the UPR by H_2O_2 in HeLa cells: role of mild thermotolerance

The exposure to milder conditions of stress can often induce cellular defenses to enable the cell to survive. Therefore, the ability of H_2O_2 to activate the UPR, under mild conditions, was evaluated in non-thermotolerant HeLa cells. The activation status of the ER stress sensors PERK, ATF6 and IRE1α was evaluated, as well as expression of chaperone Bip (Figs. 5-9). The treatment of cells with H_2O_2 (15-50 μM) for 15 min caused significant and dose-dependent activation (phosphorylation) of PERK (Fig. 5A, 5B), eIF2α (Fig. 6A, 6B) and IRE1α (Fig. 7A, 7B), relative to untreated controls. H_2O_2 caused cleavage of ATF6, which is shown by a decrease in protein expression of its 90 kDa native form (Fig. 8A, 8B) and an increase in its 36 kDa cleavage fragment (Fig. 8C, 8E), relative to untreated controls. H_2O_2 induced protein expression of Bip after 15 min (Fig. 9A, 9C). These early ER stress responses were essentially lost after a longer 30 min treatment with H_2O_2 (data not shown). Together, these results show that exposure to mild stress induced by H_2O_2 activates the 3 branches of the UPR in HeLa cells.

Subsequently, the ability of mild thermotolerance (40°C, 3 h) to modulate activation of the UPR by H_2O_2 was determined (Figs 5-9). As mentioned above (section 3.2), non-

phosphorylated and phosphorylated forms of PERK (Fig. 2) and eIF2α (Fig. 3) were higher in thermotolerant versus non-thermotolerant cells. However, when thermotolerant cells were treated with H_2O_2, there were no significant changes in levels of p-PERK (Fig. 5B 5C), PERK (Fig. 5C, 5D), p-eIF2α (Fig. 6B, 6C) and eIF2α (Fig. 6C, 6D), compared to untreated non-thermotolerant controls. In general, levels of these proteins were higher in H_2O_2-treated thermotolerant cells compared to their non-thermotolerant counterparts (Fig. 5B, 5D, 6B, 6D), except for PERK expression at 50 μM H_2O_2. For IRE1α, H_2O_2-induced increases in expression of p-IRE1α and IRE1α (Fig. 7A) were inhibited in thermotolerant cells (Fig. 7B, 7C, 7D). For ATF6, there was a dose-dependent increase in expression of the 90 kDa form when thermotolerant cells were treated with 15-50 μM H_2O_2 (Fig. 8A, 8B). Levels of ATF6 were significantly higher in H_2O_2-treated thermotolerant cells, compared to non-thermotolerant cells. The generation of the cleaved form of ATF6 (36 kDa) by H_2O_2 (Fig. 8C) was partially decreased in thermotolerant cells (Fig. 8D, 8E). Levels of Bip increased slightly in H_2O_2-treated thermotolerant cells (Fig. 9B, 9C). Together, these results show that the PERK/eIF2α branch of the UPR remains activated in H_2O_2-treated thermotolerant cells. In addition, protein levels of ATF6 (90 kDa) were induced by H_2O_2 in thermotolerant cells, whereas cleavage of ATF6 and activation of IRE1α by the oxidant were decreased.

H_2O_2 induces ER-mediated apoptosis in HeLa cells

H_2O_2 up-regulates pro-apoptotic factor CHOP: effect of mild thermotolerance

Severe and prolonged ER stress generally results in cell death via apoptosis. The pro-apoptotic transcription factor CHOP (also known as growth-arrest and DNA-damage-inducible gene 153 (GADD 153)) is strongly induced in response to ER stress [Malhotra and Kaufman, 2007]. Exposure of HeLa cells to pro-oxidant H_2O_2 (25, 50 μM) for a longer time of 2 h caused a significant 1.2 to 1.6-fold increase in CHOP expression compared to untreated control cells (Fig. 10A, 10B). Mild thermotolerance (40°C) completely inhibited the induction of CHOP expression by H_2O_2.

Activation of calpain by H_2O_2: role of mild thermotolerance

The alteration of ER calcium homeostasis can result in activation of cytosolic proteases known as calpains. They play a major role in ER-mediated apoptosis by processing and activating the ER initiators caspase-12 and caspase-4 [Nakagawa and Yuan, 2000; Tan et al, 2006]. We therefore determined if calpains are involved in ER-mediated apoptosis during exposure of HeLa cells to H_2O_2. Calpain activity is regulated by its endogenous inhibitor, calpastatin. Treatment of cells with H_2O_2 resulted in calpastatin cleavage (Fig. 11). Levels of the 110 kDa cleaved fragment increased by 1.45 to 1.8-fold as a function of H_2O_2 concentration, compared to untreated control cells (Fig. 11A, 11B). Calpastatin cleavage by H_2O_2 was significantly attenuated in mild thermotolerant cells (Fig. 11A, 11B). As a consequence, there was a significant increase in enzymatic activity of calpains upon treatment with H_2O_2 (Fig. 12A). Active calpains are found predominantly at the plasma membrane. Indeed, increased protein levels of calpain were detected in microsomal fractions (Fig. 12B, 12C) of H_2O_2-treated cells, along with a corresponding decrease in levels in cytosolic fractions (Fig. 12D, 12E). Mild thermotolerance significantly diminished H_2O_2-induced calpain activation (Fig. 12A), and its translocation to the plasma membrane (Fig. 12B-12E).

Roles of calcium, calpain and caspase-7 in activation of ER caspases-4 and -12 during exposure to H_2O_2: protective role of mild thermotolerance

Caspase-12 (mice) and -4 (human) have been proposed as caspases that initiate ER stress-induced apoptosis [Fulda et al, 2010; Przybytkowski et al, 1986]. Pro-caspase-12 is localized at the cytosolic side of the ER membrane and can be activated through several mechanisms, including cleavage by m-calpain and by caspase-7 [Breckenridge et al, 2003; Nakagawa and Yuan, 2000; Tan et al, 2006; Boyce and Yuan, 2006]. Treatment of HeLa cells with H_2O_2 for 2 h led to a significant increase in enzymatic activities of caspase-4 (Fig. 13A) and caspase-12 (Fig. 13B). H_2O_2 caused a significant increase in levels of the active cleavage fragments of caspase-4 (20 kDa) (Fig. 13C, 13D) and

caspase-12 (33 kDa) (Fig. 13E, 13F). The activation of caspases-4 and -12 was inhibited in mild thermotolerant (40°C) cells (Fig. 13A-13F).

Subsequently, the upstream factors responsible for activation of ER caspases were determined. During ER stress, caspase-7 associates with procaspase-12 and can cleave the pro-domain to initiate the processing of caspase-12 [Fan et al, 2005]. A significant increase in enzymatic activity of caspase-7 activation was seen in HeLa cells exposed to H_2O_2 for 2 h (Fig. 14A). No activity was detected after a shorter 1 h exposure to H_2O_2. Mild thermotolerance significantly attenuated H_2O_2-induced caspase-7 activation (Fig. 14A). The activation of caspase-4 (Fig. 14B) and caspase-12 (Fig. 14C) was significantly inhibited by a calcium chelator (BAPTA-AM), and by inhibitors of calpain and caspase-7. This confirms the roles of calcium, calpain and caspase-7 as activating factors of the ER caspases-4 and -12.

Once activated, caspase-12 can activate caspase-9, which in turn can catalyze cleavage of procaspase-3 [Morishima et al, 2002]. The roles of caspase-12 and caspase-4 in the activation of caspase-9 and caspase-3 in H_2O_2-treated cells were evaluated by means of inhibitors of caspase-4 and caspase-12. The activation of caspase-9 was decreased partially, but not completely, by inhibitors of caspase-4 and caspase-12 (Fig. 15A). These results suggest that caspase-12 and -4 can directly activate caspase-9, without the involvement of cytochrome c and mitochondria, during H_2O_2-induced apoptosis. In a similar manner, the inhibitors of caspase-4 and -12 partially inhibited the activation of executioner caspase-3 by H_2O_2 (Fig. 15B). Furthermore, BAPTA-AM and the calpain inhibitor also caused partial inhibition of caspase-3 activation by H_2O_2 (Fig. 15B). H_2O_2-induced apoptosis (chromatin condensation) was also partially decreased by BAPTA-AM, and inhibitors of calpain and caspases-7, -4 and -12 (Figure 15C). These findings suggest that the activation of caspase-3 and the induction of apoptosis by H_2O_2 are

dependent, in part, on the disruption of calcium homeostasis and apoptosis mediated by the ER.

Role of the ER stress response in resistance to H_2O_2-induced apoptosis

We subsequently determined if a causal connection exists between the ER stress response and resistance to H_2O_2-induced apoptosis. Therefore, levels of PERK were decreased to evaluate whether the resistance to H_2O_2-induced apoptosis seen following exposure to mild hyperthermia could be inhibited. Transfection of cells with a siRNA targeted against PERK led to a decrease in protein levels of PERK to 60% of control levels (Fig. 16A, 16B). Cells with control or decreased levels of PERK were then made thermotolerant or non-thermotolerant, and exposed to 50 μM H_2O_2 for 3 h. Knock down of PERK had little effect on H_2O_2-induced apoptosis in non-thermotolerant cells. However, in thermotolerant cells with decreased levels of PERK, there was partial reversal of resistance H_2O_2-induced apoptosis, compared to scrambled siRNA controls (Fig. 16C, 16D). This shows that the ER stress response plays a partial role in resistance to H_2O_2-induced apoptosis following mild heat preconditioning.

DISCUSSION

Induction of the UPR by mild thermotolerance (40°C) in HeLa cells

This study shows that mild thermotolerance developed at 40°C can elicit the ER stress response. Mild heat stress enhanced the pro-survival effects of the PERK/eIF2α branch of the UPR. Adaptive responses induced by pre-exposure of cells and tissues to low levels of stresses often induce cellular defenses including stress proteins such as Hsps, antioxidants (SOD, catalase), anti-apoptosis proteins (e.g. Bcl-2, inhibitor of apoptosis proteins (IAPs), cellular FLICE-inhibitory protein (cFLIP)) and damage repair molecules, as well as survival signaling pathways involving extracellular regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3-K)/Akt [Fulda et al, 2010; Portt et al,

2011; Davies, 2000]. In addition, the ER plays a role as an adaptive survival response mediated by three ER membrane receptors PERK, ATF6 and IRE1α [Faitova et al, 2006].

In HeLa cells, mild heat stress (40°C, 3 h) triggered the induction of Hsps [Bettaieb and Averill-Bates, 2008] and antioxidants (SOD, catalase, glutathione) [Pallepati and Averill-Bates, 2010]. This study shows further that mild thermotolerance (40°C) can activate another cell survival mechanism, the PERK/eIF2α branch of the UPR. The phosphorylation of eIF2α allows translation of ATF4, which induces the transcription of survival genes involved in amino acid metabolism, redox reactions, the stress response, and protein secretion [Szegezdi et al, 2006]. Once activated, PERK can mediate an anti-apoptotic response by phosphorylating nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that promotes cell survival in response to ER stress [Cullinan et al, 2003]. The phosphorylation of Nrf2 by PERK activates the transcription of protective genes containing antioxidant responsive elements (AREs) such as NAD (P)H:quinone oxidoreductase 1, heme oxygenase 1, glutathione S-transferase and the rate limiting enzyme in glutathione biosynthesis, γ-glutamylcysteine synthetase (γ-GCS) [Venugopal and Jaiswal, 1998; Wild et al, 1999; He et al, 2001; Nguyen et al, 2005]. Interestingly, mild thermotolerance increased protein expression of γ-GCS and glutathione levels in HeLa cells [Pallepati and Averill-Bates, 2010], which could be a consequence of PERK activation. Enhanced GSH content observed in thermotolerant cells could scavenge mitochondrial ROS and prevent the opening of the mitochondrial permeability transition pore, cytochrome c release and caspase-9 activation (Franco and Cidlowski, 2009).

Lower doses of H_2O_2 activate the UPR while higher doses cause apoptosis through the ER

ROS cause protein damage, which could lead to accumulation of misfolded proteins in the ER lumen. Mild oxidative stress (H_2O_2 (15-50 μM, 15 min) activated the three ER stress sensors, PERK, IRE1α and ATF6 in HeLa cells. The activation of these sensors

increases the protein folding capacity of the cell by promoting gene transcription for ER chaperones and ER-associated degradation of damaged proteins [Malhotra and Kaufman, 2007; Rasheva and Domingos, 2009]. H_2O_2 (50 to 500 μM, 24 h) caused ER stress (induction of GRP78/Bip, p-PERK and p-eIF2α) in human oral keratinocytes and oral cancer cells [Min et al, 2008]. In mesenchymal stem cells, H_2O_2 (120 μM, 6 to 24 h) caused an increase in the expression of Bip [Wei et al, 2010].

This study shows that the ER stress survival response predominates under milder exposure conditions (15 min) to H_2O_2 (15-50 μM), whereas apoptosis predominates during more severe oxidative stress (1-3 h). ER-mediated apoptosis involved increased expression of CHOP, cleavage of calpastatin, and activation of calpain, caspase-4 and -12. Caspase-4 cleavage and up-regulation of CHOP was induced by peroxide (200 to 500 μM, 24 h) in human oral keratinocytes and oral cancer cells [Min et al, 2008]. H_2O_2 (120 μM, 6 to 24 h) caused cleavage of procaspase-12 in mesenchymal stem cells [Wei et al, 2010]. The pro-oxidant tert-butylhydroperoxide (tBOOH) caused calpain-mediated cleavage of procaspase-12 at the ER and its translocation to the nucleus, where increased caspase-12 activity was found, in rat hepatocytes [Haidara et al, 2008].

The exact mechanisms that regulate apoptosis through the ER are not well understood. Several different pathways have been implicated including the caspase-12/caspase-4 pathway, CHOP, and IRE1-JNK pathways [Fulda et al, 2010]. Although caspases-12 and -4 have been implicated in ER stress-induced apoptosis, the events responsible for their activation remain ill-defined. This study shows that calcium, calpain and caspase-7 are among the initiating factors for the caspase-12/caspase-4 pathway in H_2O_2-induced apoptosis through the ER in HeLa cells.

The role of caspase-12 in ER-mediated apoptosis is well established in mice [Nakagawa et al, 2000]. However, its role in apoptosis of human cells is unresolved,

since the human caspase-12 gene contains several inactivating mutations [Fischer et al, 2002]. Despite this, caspase-12 activation has been detected in several human cell lines, including retinal pigment epithelial (ARPE-19) [Luthra et al, 2006], adenocarcinomic alveolar basal epithelial (A549) [Bitko and Barik, 2001], bronchial epithelial [Kerbiriou et al, 2009], embryonic kidney (HEK293T) [Yoneda et al, 2001] and HeLa cells [Won et al, 2010; Hu et al, 2005]. Our results are in concordance with these studies. Once activated, caspase-12 causes cytochrome c-independent caspase-9 activation, followed by caspase-3 activation [Morishima et al, 2002; Rao et al, 2004]. The partial attenuation of caspase-9 activation by a caspase-12 inhibitor suggests that caspase-9 can be activated by alternative pathways in H_2O_2-treated HeLa cells. Indeed, H_2O_2 caused caspase-9 activation through release of cytochrome c from mitochondria in HeLa cells [Pallepati and Averill-Bates, 2010]. In mesenchymal stem cells, H_2O_2 induced apoptosis through both mitochondrial and ER pathways [Wei et al, 2010].

Caspase-4 is considered to fulfill the function of caspase-12 in human cells [Hitomi et al, 2004; Kim et al, 2006]. The activation of caspase-4 was mediated by calcium and calpain during ER stress caused by tunicamycin and thapsigargin in SK-N-SH cells [Matsuzaki et al, 2010]. However, little information is available about putative caspase-4 substrates. It was reported that caspase-4 can cleave actin, ataxin and the 65 kDa subunit of splicing factor U2AF [Agard et al, 2010]. The inhibition of caspase-9 activation by a caspase-4 inhibitor in our study suggests that caspase-4 cleaved caspase-9 in a cytochrome c-independent manner, similar to caspase-12. The over-expression of caspase-4 in COS-7 cells resulted in caspase-9 cleavage in the absence of cytochrome c release from mitochondria [Yukioka et al, 2008].

The transcription of CHOP can be induced by the three ER sensors, but the PERK/eIF2α branch of the UPR is required for up-regulation of CHOP protein expression [Malhotra and Kaufman, 2007; Szegezdi et al, 2006]. However, the details of

the apoptosis pathway downstream of CHOP are not well understood. CHOP appears to be linked to apoptosis by down-regulating Bcl-2 expression [McCullough et al, 2001] and inducing the expression of BH3-only protein Bim [Puthalakath et al, 2007]. Transfection studies done in HeLa cells show that, CHOP downregulates Bcl-2 expression by inhibiting bcl2 transcription (McCullough et al, 2001).

Mild thermotolerance as an adaptive apoptosis-resistant phenotype

This study shows that mild thermotolerance developed at 40°C inhibited all of the pro-apoptotic events induced by H_2O_2 at the level of the ER, as well as clonogenic cell killing. Furthermore, the PERK/eIF2α branch of the UPR contributes in part to this apoptosis-resistant phenotype in HeLa cells. However, other mechanisms must also contribute to the apoptosis-resistant phenotype and these could involve increased levels of Hsps and antioxidants [Pallepati and Averill-Bates, 2010; Bettaieb and Averill-Bates, 2008]. Mild hyperthermia induces catalase and peroxidase pathways that metabolize H_2O_2 [Pallepati and Averill-Bates, 2010]. These pathways could account for the resistance to H_2O_2 following mild hyperthermia, independent of the ER stress response. The ability of Hsps to inhibit apoptosis through the mitochondrial and death receptor pathways has been well characterized [Beere, 2004]. Hsp72 was shown to bind to IRE1α, allowing PC-12 cells to adapt to ER stress by enhancing the pro-survival effects of the IRE1α/XBP1 branch of the UPR [Gupta et al, 2010]. Hsp27 and Hsp70 can both maintain cellular redox homeostasis. Hsp27 is able to increase glutathione levels [Arrigo et al, 2005], reduce cytosolic ROS levels [Wyttenbach et al, 2002], increase glucose 6-phosphate dehydrogenase activity [Préville et al, 1999] and/or decrease intracellular iron levels [Arrigo et al, 2005].

CONCLUSION

This study improves understanding about the activation of ER stress and ER-mediated apoptosis by oxidative stress. Moreover, the adaptive response (UPR) dominates during milder oxidative stress, whereas ER-mediated apoptosis occurs during more severe stress. Furthermore, it advances knowledge about the protective effect of adaptive responses induced by mild stresses, such as fever temperatures, against the induction of apoptosis by oxidative stress through the ER.

ACKNOWLEDGMENTS

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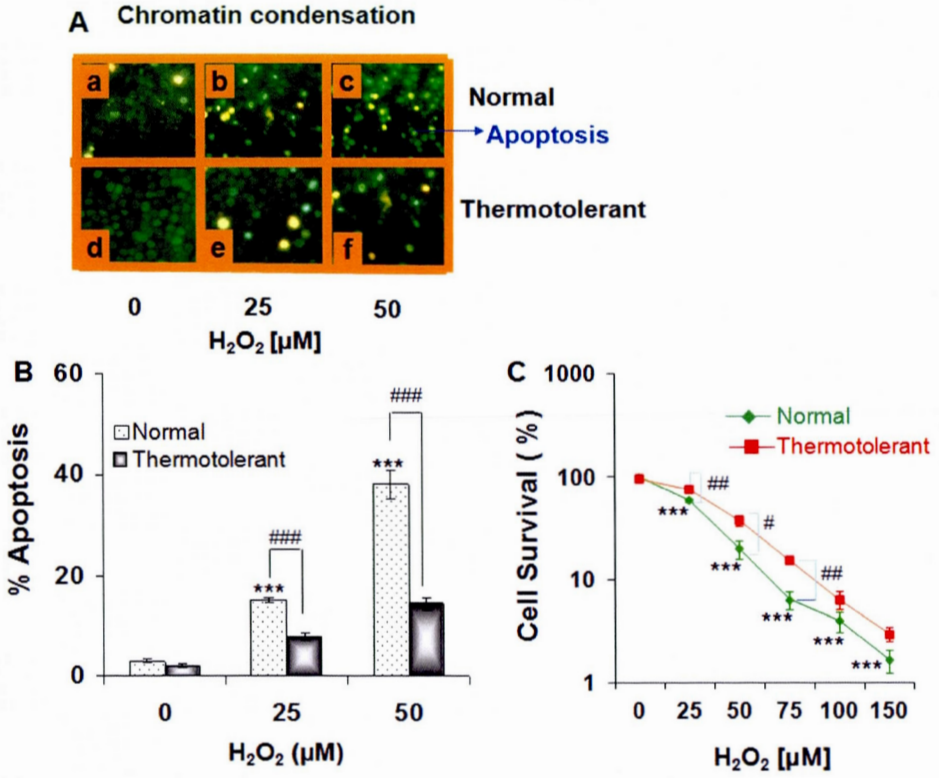

Figure 1 Mild heat pretreatment at 40°C causes resistance to H_2O_2–induced apoptosis and clonogenic cell killing. (A) (a to c) Normal (non-thermotolerant) (37°C, 3 h) and (d to f) thermotolerant (40°C, 3 h) HeLa cells (10^6/ml) were incubated with H_2O_2 concentrations of 0 μM (a, d), 25 μM (b, e), and 50 μM (c, f) for 3 h. (B) The fractions of apoptotic (Hoechst 33258) cells are given relative to the total number of cells (magnification 320 X). Necrotic cells, stained with PI, were < 5%. (C) Clonogenic cell survival is shown for H_2O_2-treated thermotolerant versus normal cells. The control value represents 10^5 cells and was normalized to represent 100% cell survival. Data represent means and SEM from three independent experiments performed with multiple estimations per point. ***P<0.001 indicates a statistically significant difference between treatment with H_2O_2 and the untreated control. ##P<0.01 or ###P<0.001 indicates a statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

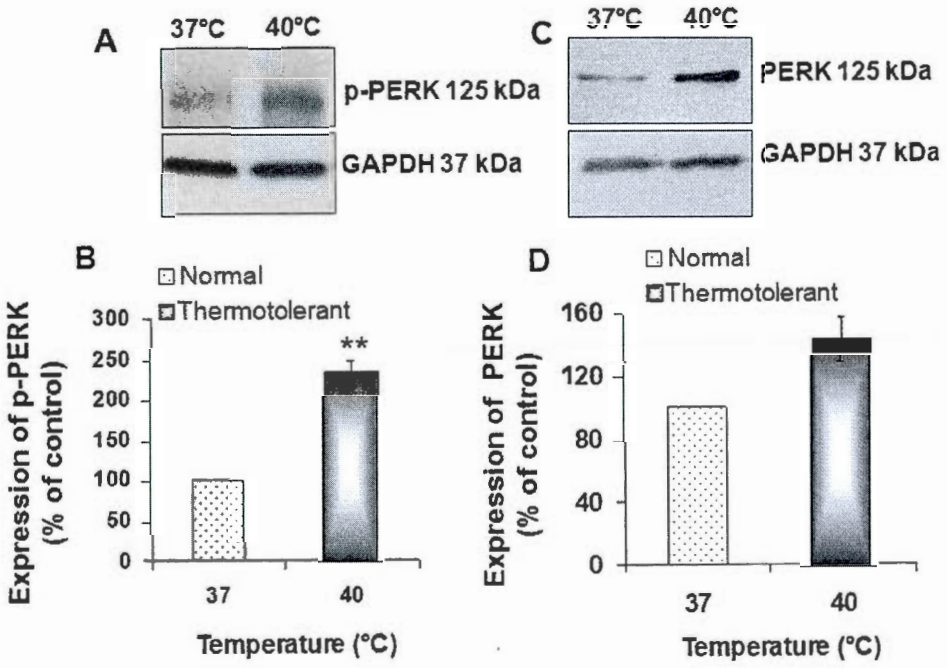

Figure 2 Induction and activation of PERK in cells at a mild temperature of 40°C. Western blot analysis of (A) p-PERK and (C) PERK in whole cell lysates of non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) HeLa cells. Representative blots are from four individual experiments. Densitometric analyses for expression of (B) p-PERK and (D) PERK are relative to untreated controls, designated as 100%, using GAPDH as loading control. Data represent means and SEM from four independent experiments performed with multiple estimations per point. **P<0.01: statistically significant difference compared to the untreated control.

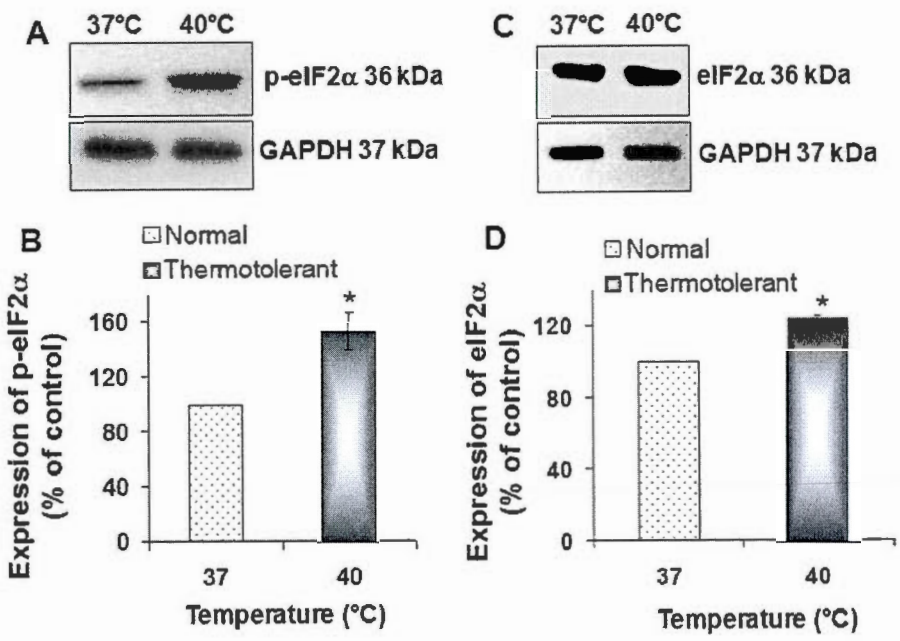

Figure 3 Mild thermal stress (40°C) causes induction and phosphorylation of eIF2α. Immunodetection of (A) p-eIF2α and (C) eIF2α in whole cell lysates of non-thermotolerant and thermotolerant cells, using GAPDH as loading control. Representative blots are from three individual experiments. Densitometric analyses for expression of (B) p-eIF2α and (D) eIF2α (±SEM) are relative to untreated controls (100%). *P<0.05: statistically significant difference compared to the untreated control.

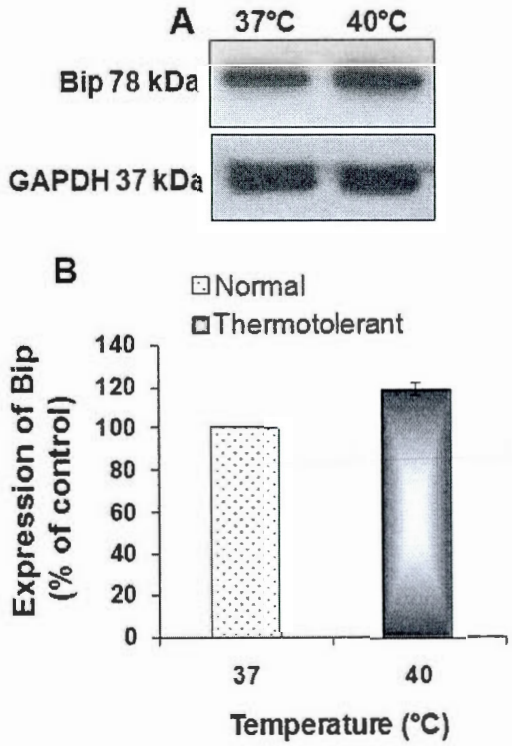

Figure 4 Protein expression of chaperone protein Bip (GRP78) in non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) cells. (A) Levels of Bip (78 kDa) in whole cell lysates were analyzed by Western blotting. A representative blot is shown from three individual experiments. (B) Densitometric analysis for Bip expression (±SEM) is relative to the untreated control (100%).

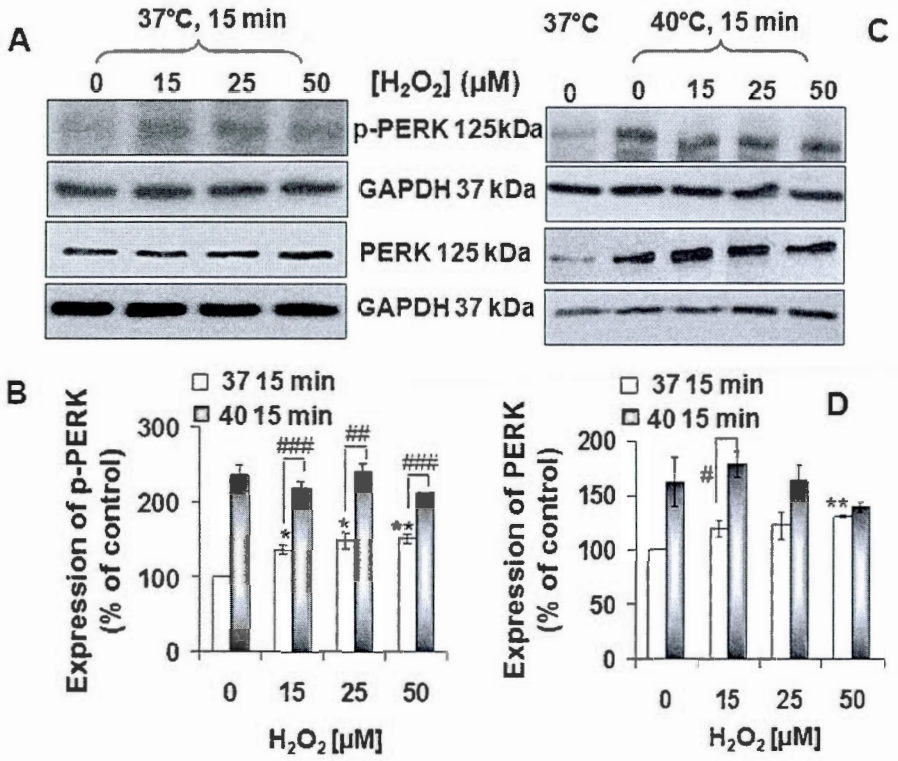

Figure 5 H_2O_2 causes activation of PERK: role of mild thermotolerance. Non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) HeLa cells were incubated for 15 min at 37°C with H_2O_2 (0-50 μM) in D-MEM containing 10% FBS. Representative Western blots are for p-PERK (n=4) and PERK (n=3) in (A) non-thermotolerant and (C) thermotolerant cells (+ normal control at 37°C), using GAPDH as loading control. Densitometric analyses for expression of (B) p-PERK and (D) PERK (±SEM) are relative to the untreated non-thermotolerant controls at 37°C (100%). *P<0.05 or **P<0.01: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05, ##P<0.01, or ###P<0.001: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

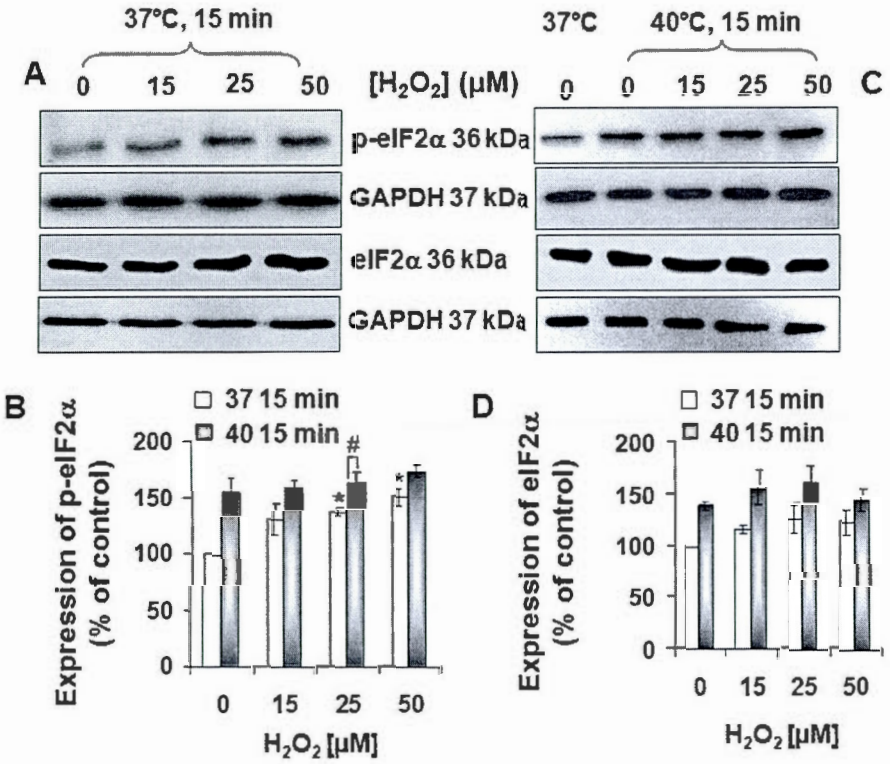

Figure 6 H_2O_2 causes eIF2α phosphorylation: role of thermotolerance at 40°C. Non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) cells were incubated for 15 min at 37°C with H_2O_2 (0-50 μM). Representative Western blots from three individual experiments are for p-eIF2α and eIF2α in (A) non-thermotolerant and (B) thermotolerant cells (+ normal control at 37°C), using GAPDH as loading control. Densitometric analyses for expression of (B) p-eIF2α and (D) eIF2α (±SEM) are relative to the untreated non-thermotolerant controls at 37°C (100%). *P<0.05: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

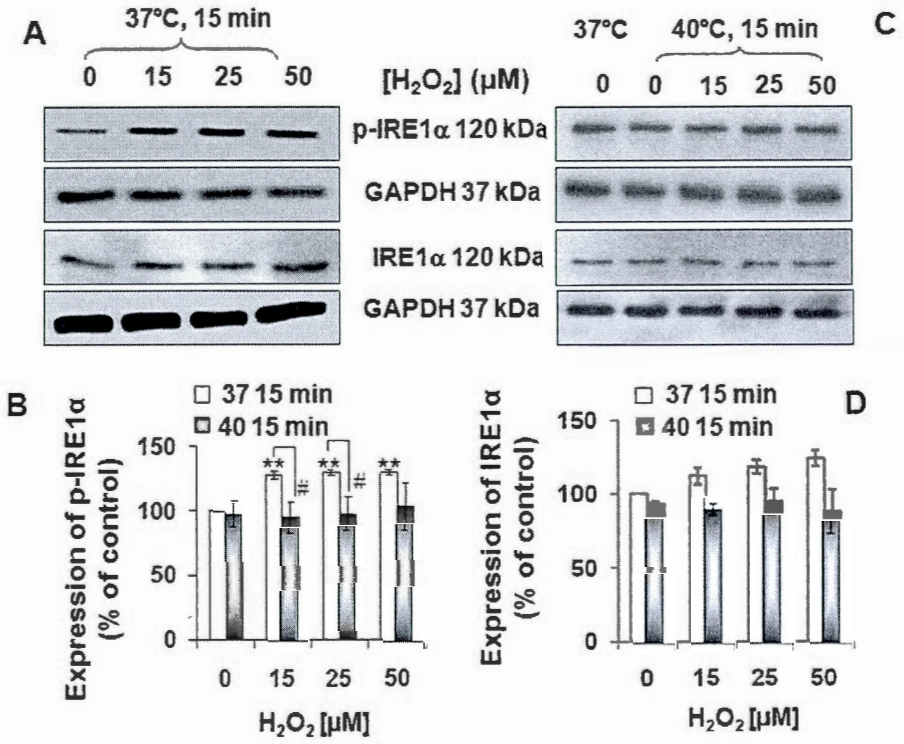

Figure 7 H_2O_2 causes IRE1α activation: inhibition in thermotolerant cells. Non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) cells were exposed to H_2O_2 (0-50 μM) for 15 min. Representative Western blots for p-IRE1α and IRE1α in (A) non-thermotolerant and (C) thermotolerant cells (+ normal control at 37°C) are from three individual experiments, using GAPDH as loading control. Densitometric analyses for expression of (B) p-IRE1α and (D) IRE1α (±SEM) are relative to untreated non-thermotolerant controls at 37°C (100%). **P<0.01: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

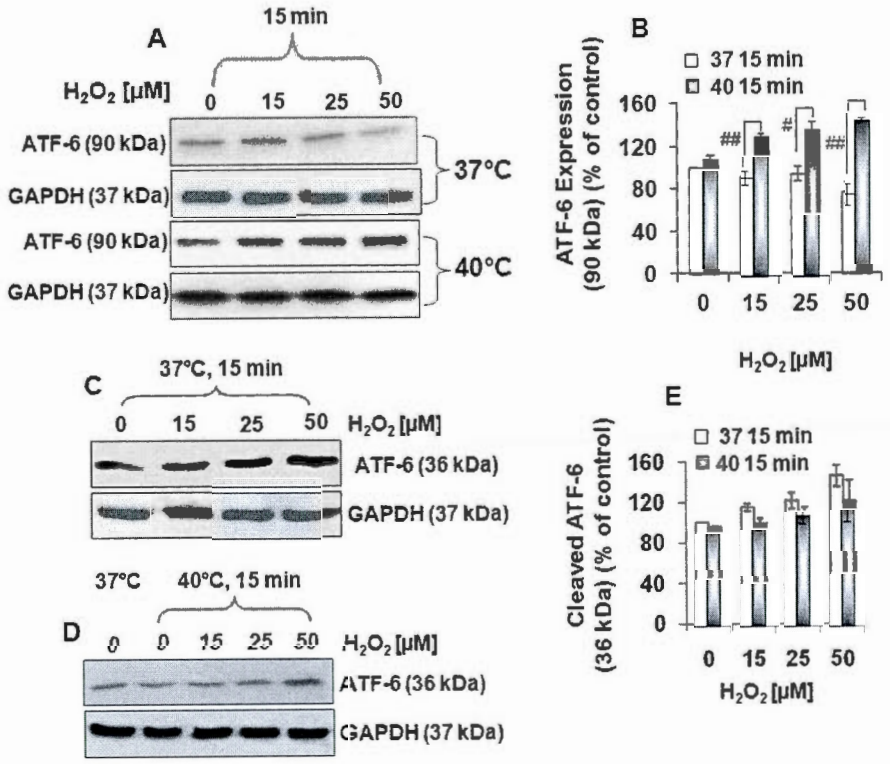

Figure 8 H_2O_2 causes cleavage of ATF6: role of mild thermotolerance. (A, C) Non-thermotolerant (37°C, 3 h) and (A, D) thermotolerant (40°C, 3 h) cells were incubated for 15 min at 37°C with H_2O_2 (0-50 μM) in D-MEM containing 10% FBS. Representative Western blots for (A) ATF6 (90 kDa) and (C, D) cleaved ATF6 (36 kDa) are from three independent experiments, using GAPDH as loading control. Densitometric analyses for expression of (B) ATF6 (90 kDa) and (E) cleaved ATF6 (36 kDa) (±SEM) are relative to the untreated non-thermotolerant controls at 37°C (100%). #P<0.05 or ##P<0.01: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

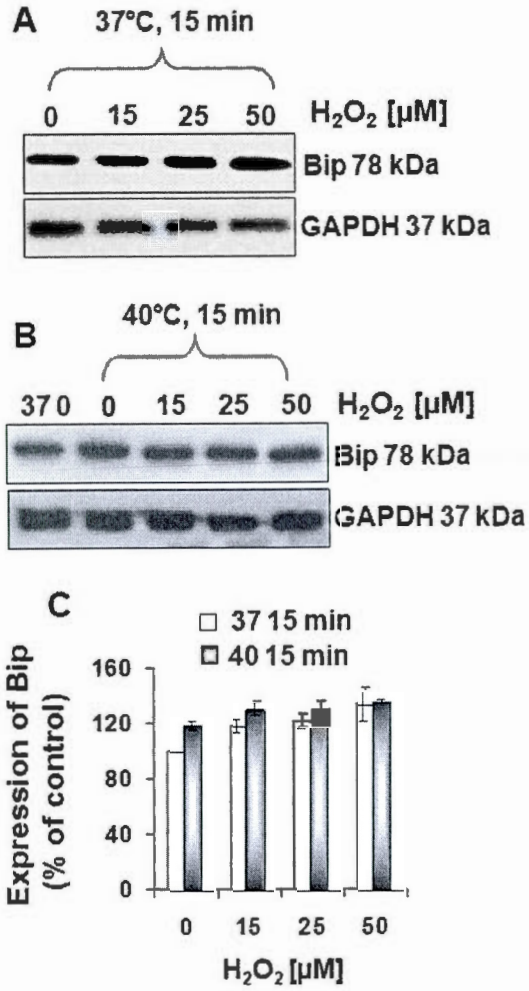

Figure 9 Effect of H_2O_2 on expression of Bip. HeLa cells were incubated with H_2O_2 (0-50 μM) for 15 min. Representative Western blots for Bip (78 kDa) in (A) non-thermotolerant and (B) thermotolerant cells (+ normal control at 37°C) are from three individual experiments, relative to GAPDH. (C) Densitometric analysis for Bip expression (±SEM) is relative to the untreated non-thermotolerant control (100%).

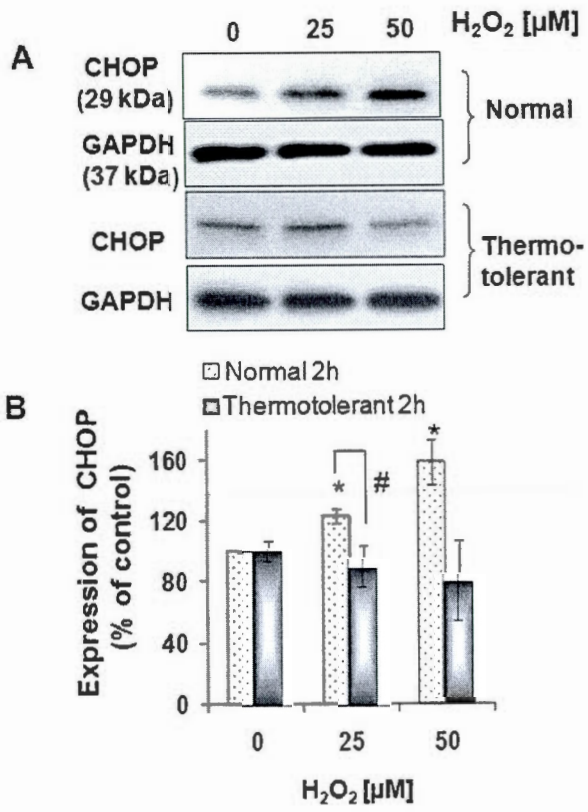

Figure 10 H_2O_2 increases CHOP expression in HeLa cells: protective role of thermotolerance (40°C). Non-thermotolerant and thermotolerant cells were treated with H_2O_2 (0-50 µM) for 2 h. (A) A representative Western blot is shown for CHOP (28 kDa) expression from four independent experiments, using GAPDH as loading control. (B) Densitometric analysis for CHOP expression (±SEM) is relative to the untreated non-thermotolerant control (100%). *P<0.05: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

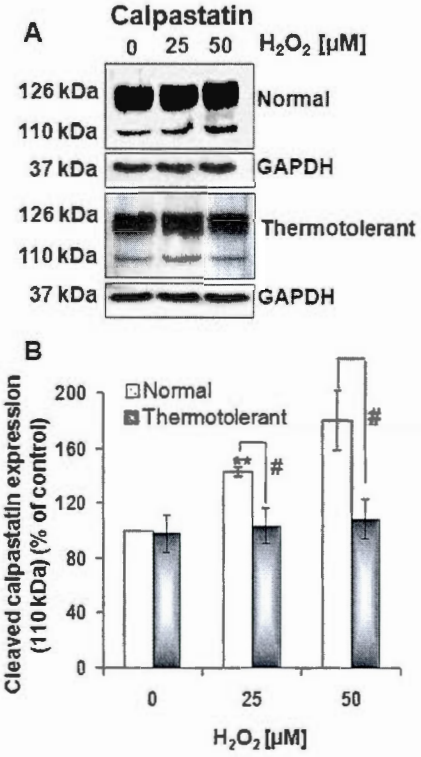

Figure 11 H_2O_2 causes calpastatin cleavage: inhibition by mild thermotolerance. Non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) cells were exposed to H_2O_2 (0-50 μM) for 1 h at 37°C in D-MEM containing 10% FBS. (A) A representative Western blot for calpastatin (126 kDa) and its cleavage fragment (110 kDa) is shown from three independent experiments. (B) Densitometric analysis for expression of the cleaved calpastatin fragment (110 kDa) (±SEM) is relative to the untreated non-thermotolerant control at 37°C (100%). **P<0.01: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

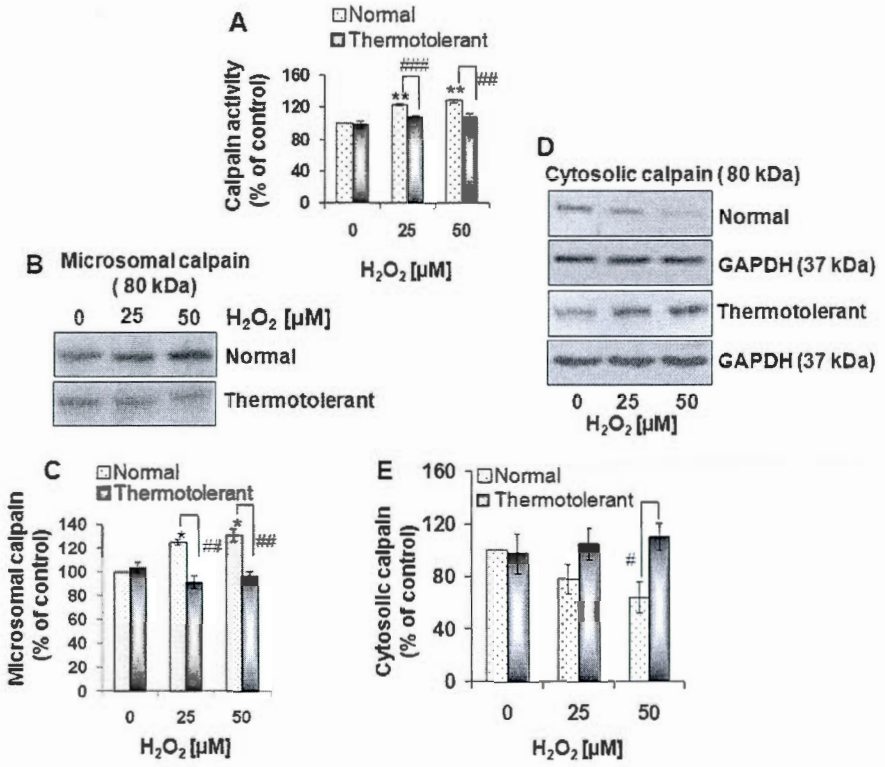

Figure 12 H_2O_2 induces calpain activation and translocation to the plasma membrane: abrogation by mild thermotolerance. Non-thermotolerant and thermotolerant HeLa cells were exposed to H_2O_2 (0-50 μM) for 1 h at 37°C in D-MEM containing 10% FBS. (A) Calpain activity in cell lysates is relative to the untreated non-thermotolerant control at 37°C (100%). Western blots for μ-calpain expression (80 kDa) in (B) microsomal and (D) cytosolic fractions are representative of four independent experiments. Calpain expression (±SEM) in microsomal (C) and cytosolic (E) fractions was analyzed by densitometry and is relative to untreated non-thermotolerant controls at 37°C (100%). *P<0.05 or **P<0.01: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05, ##P<0.01, or ###P<0.001: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

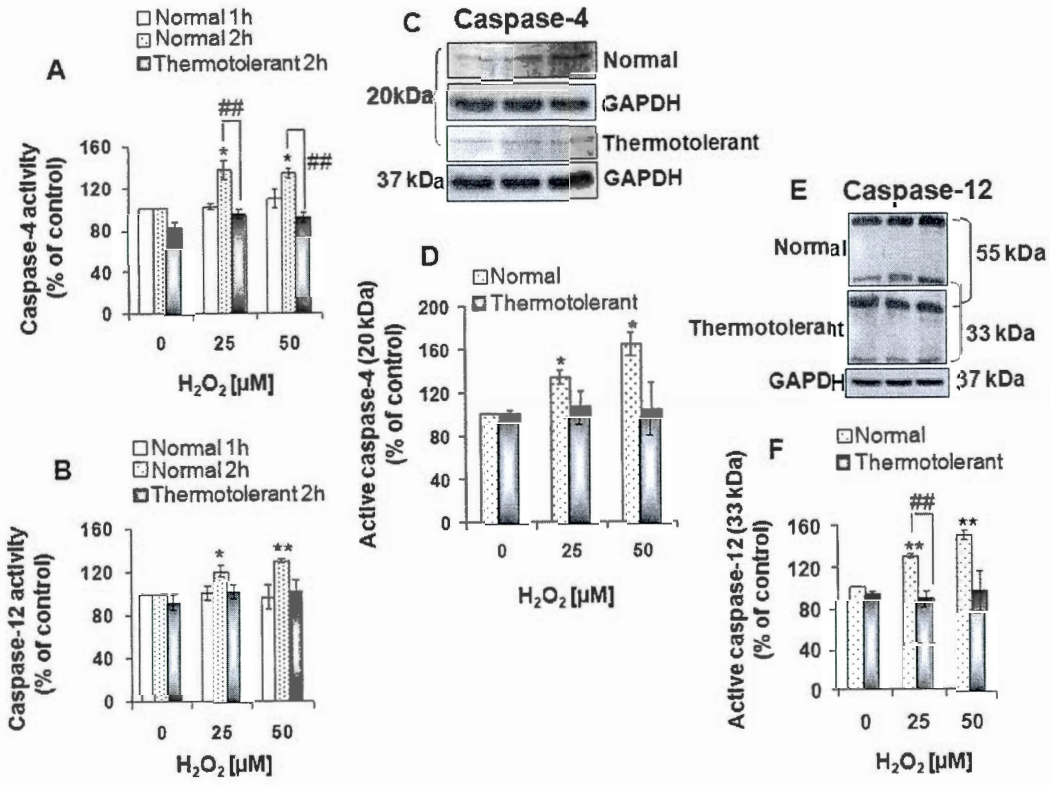

Figure 13 Activation of caspase-4 and caspase-12 by H_2O_2: inhibition by mild thermotolerance. Non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) HeLa cells were exposed to H_2O_2 (0-50 μM) for (A, B) 1 h or (A-F) 2 h at 37°C. Enzymatic activities of (A) caspase-4 and (B) caspase-12 are relative to untreated non-thermotolerant controls at 37°C (100%). Data represent means and S.E. from three independent experiments. Representative Western blots for expression of (C) caspase-4 and (E) caspase-12 are from three independent experiments, relative to GAPDH. Densitometric analyses for expression of the cleavage fragments of (D) caspase-4 (p20) and (F) caspase-12 (p33) (±SEM) are given relative to untreated non-thermotolerant controls at 37°C (100%). *P<0.05 or **P<0.01: statistically significant difference between treatment with H_2O_2 and the untreated control. ##P<0.01: statistically significant difference between treatments (± thermotolerance) for a given concentration of H_2O_2.

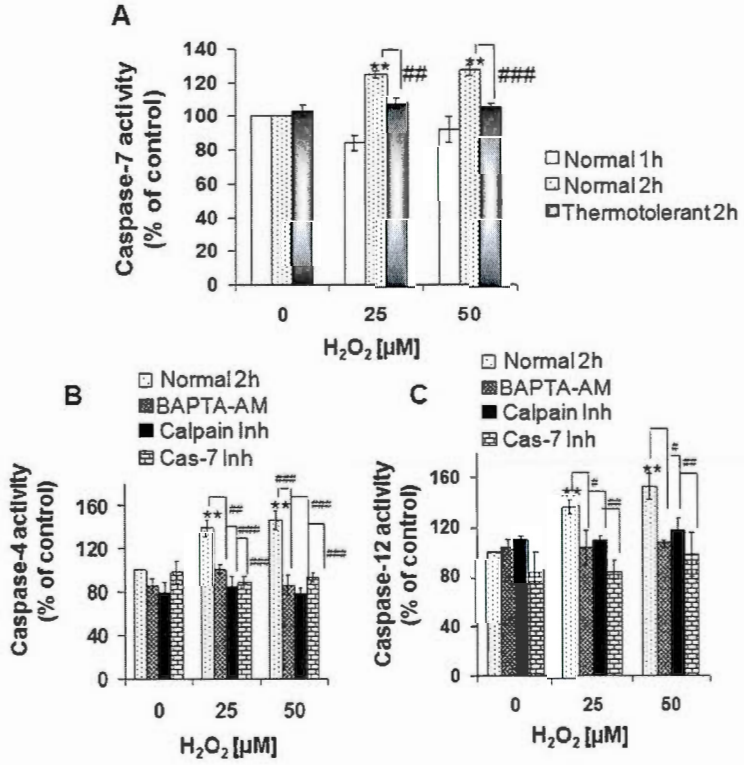

Figure 14 Roles of calcium, calpain and caspase-7 in H_2O_2-induced activation of caspase-4 and caspase-12. Non-thermotolerant (37°C, 3h) and thermotolerant (40°C, 3 h) HeLa cells (1 x 10^6/ml) were exposed to H_2O_2 (0-50 µM) for 1 or 2 h at 37°C. (A) Caspase-7 activity in cell lysates is relative to the untreated non-thermotolerant control at 37°C (100%). Non-thermotolerant (37°C, 3 h) and thermotolerant (40°C, 3 h) cells, with or without pretreatment (1 h) with inhibitors of calpain (20 µM), caspase-7 (50 µM), or a calcium chelator (BAPTA-AM, 50 µM), were exposed to H_2O_2 (0-50 µM) for 2 h. Enzymatic activities of (B) caspase-4 and (C) caspase-12 are given relative to untreated non-thermotolerant controls at 37°C (100%). Data represent means and SEM from three independent experiments performed with multiple estimations per point. **P<0.01: statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05, ##P<0.01 or ###P<0.001: statistical difference between treatments (A) (±thermotolerance) or (B, C) (± inhibitor) for a given concentration of H_2O_2.

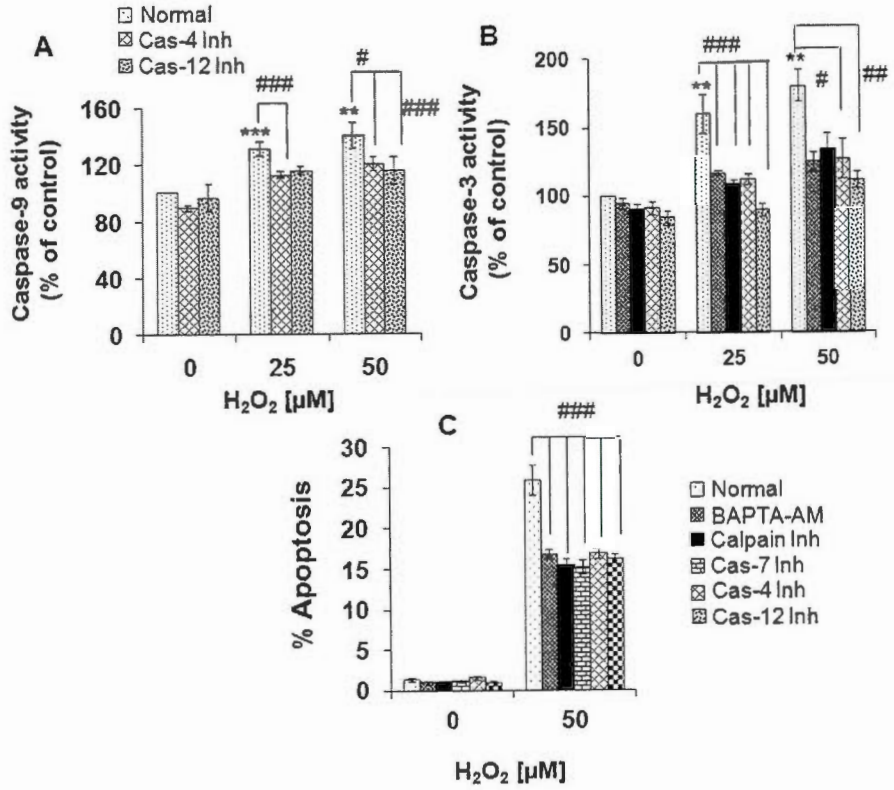

Figure 15 H_2O_2-induced activation of caspase-9, caspase-3 and apoptosis is diminished by apoptotic protease inhibitors. Normal HeLa cells (37°C), with or without pretreatment (1 h) with inhibitors of caspase-4 (20 μM), caspase-12 (10 μM), caspase-7 (50 μM) or calpain (20 μM), or a calcium chelator (BAPTA-AM, 50 μM), were exposed to H_2O_2 (0-50 μM) for 2 h (caspases) or 3 h (apoptosis) at 37°C. Enzymatic activities of (A) caspase-9 and (B) caspase-3 are given relative to untreated non-thermotolerant controls at 37°C (100%). (C) The fractions of apoptotic (Hoechst 33258) cells are given relative to the total number of cells. Data represent means and SEM from three independent experiments. **P<0.01 or ***P<0.001 indicates a statistically significant difference between treatment with H_2O_2 and the untreated control. #P<0.05, ##P<0.01, or ###P<0.001: statistically significant difference between treatments (± inhibitor) for a given concentration of H_2O_2.

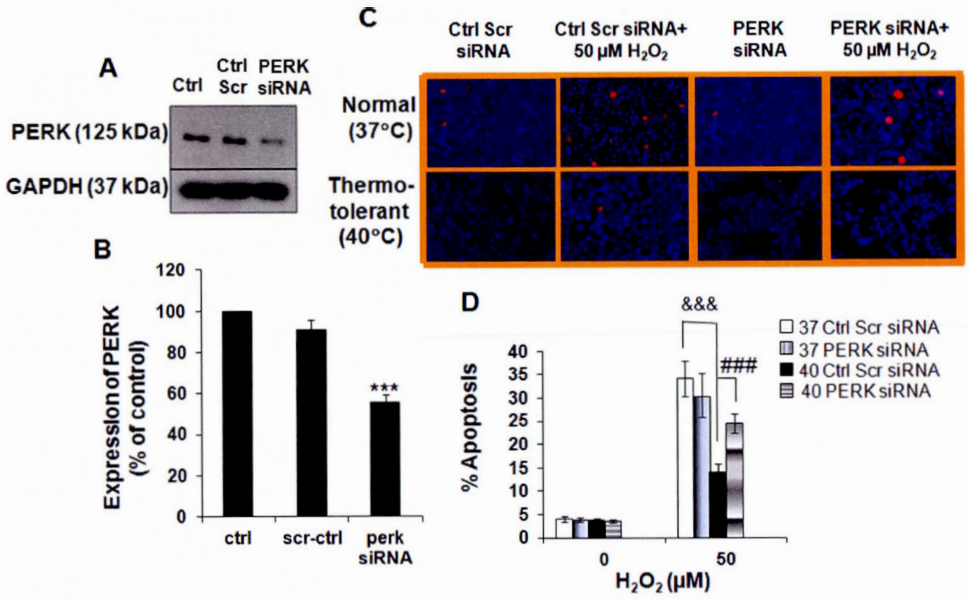

Figure 16 Knock down of PERK by siRNA causes partial reversal of resistance to H_2O_2–induced apoptosis following mild heat preconditioning. Cells were transfected with a siRNA scrambled control (Ctrl scr) and a siRNA directed against PERK (Santa Cruz) for 48 h. Non-transfected control cells are also shown. (A) Protein expression of PERK was evaluated by Western blotting and (B) analyzed by densitometry, relative to GAPDH. ***P<0.001. (C, D) Cells were transfected with siRNA PERK or siRNA scrambled control for 48 h and then rendered thermotolerant (40°C, 3 h) or non-thermotolerant (37°C, 3 h). Cells were treated with or without H_2O_2 (50 μM) for 3 h. (C) Apoptosis was detected by Hoechst staining and (D) the fraction of apoptotic cells is given. ###P<0.001 for scrambled siRNA control versus siRNA PERK at 40°C for 50 μM H_2O_2. &&&P<0.001 represents a statistically significant difference between treatments (± thermotolerance) for 50 μM H_2O_2.

CHAPTER III

DISCUSSION AND CONCLUSION

The generation of oxidative stress is a common denominator for several types of stress including IR, drugs, pollutants, herbicides, and heat shock. Several chemotherapeutic drugs (e.g., doxorubicin, cisplatin, and etoposide) induce tumour cell death by generating oxidative stress within the tumour environment although the mode of action of these durgs is different (Siddik, 2003; Bromberg et al, 2003; Fornari et al, 1994; Maanen et al, 1988), but the exact molecular mechanisms by which these cells undergo cell death are not completely understood. Mild physiological temperatures (39.5-41°C) occur during fevers. The heat shock response and thermotolerance induced at low temperatures are not well understood. Therefore, this project aimed to investigate the detailed molecular mechanisms of apoptosis induced by oxidative stress using H_2O_2 as a representative ROS, and simultaneously the protective role of mild thermotolerance against oxidative stress-induced apoptosis.

This study show that exposure of HeLa cells to the pro-oxidant H_2O_2 resulted in activation of all the three major pathways of apoptosis. H_2O_2 induced both caspase-dependent and caspase-independent mitochondrial apoptosis in HeLa cells. Caspase-independent apoptosis involved the release of AIF from mitochondria and its translocation to the nucleus (Article I: Figure 11A-D). H_2O_2 also activated the Fas death receptor pathway. H_2O_2-induced activation of initiator caspase-8 in turn resulted in activation of caspase-2 (Article II: Figure 5A-D)., which is a novel finding of this study and to our knowledge, is the first to point out the involvement of caspase-2 in apoptosis induced by H_2O_2. The results of this study also show that mild exposure (15 to 50 μM, 15 min) to H_2O_2 activates the ER stress response in HeLa cells, whereas longer exposure (1-3 h) to H_2O_2 induced ER-mediated apoptosis. Treatment with pifithrin-α decreased both

earlier and later events of apoptosis such as mitochondrial Bax translocation, FasL up-regulation, activation of caspases-8, -2, -9 and -3, and chromatin condensation, confirming the role of p53 as an upstream factor in the activation of death receptor- and mitochondrial-mediated apoptosis by H_2O_2. Mild thermotolerance (40°C, 3h) induced an increase in levels of several antioxidants (MnSOD, catalase, glutathione and γ-GCS) and ER stress proteins (PERK, p-PERK, eIF2α and p-eIF2α). All the events of the death receptor, mitochondrial and ER pathway of apoptosis induced by H_2O_2 were found to be diminished in thermotolerant cells. Mild thermotolerance even rendered HeLa resistant cells to cytotoxicity of H_2O_2.

H_2O_2 and cell death (Apoptosis vs Necrosis)

H_2O_2 is considered as an important signalling molecule based on its unique biochemical properties: H_2O_2 has a relatively long half-life, and is soluble in aqueous media (Droge, 2002). Thus, it easily diffuses to its cellular targets, triggering cell signalling pathways. The main way that H_2O_2 affects cell signalling pathways is through oxidation of specific target molecules. If the concentration of H_2O_2 is high, then these oxidation processes may lead to irreversible damage, followed by cell death, which can occur by either apoptosis or necrosis (Saito et al, 2006; Pletjushkina et al, 2006; Kroemer et al, 1998). The mode of death occurs generally depends on the severity of the insult (Golstein and Kroemer, 2007). For many years, necrosis has been considered as an accidental mode of cell death that is unregulated. The morphological changes associated with necrosis include cytoplasmic swelling, organelle breakdown and irreversible membrane rupture, which lead to release of intracellular contents that promote inflammation in surrounding tissues (Fulda et al, 2010; Van Herreweghe et al, 2010). Necrosis does not usually involve caspase activation. Necrosis can be induced by stress stimuli such as ischemia-reperfusion, inflammation, ROS and glutamate excitotoxicity.

Recent evidence suggests that necrosis is also a regulated process involving several signaling pathways (Vanlangenakker et al, 2008; Hitomi et al, 2008; Golstein and Kroemer, 2007; Duprez et al, 2009). Death receptors such as Fas, TNF-R1 and Toll-like receptors are able to trigger necrosis, in particular in the presence of caspase inhibitors (Vanlangenakker et al, 2008). The serine/threonine kinase RIP1 is an essential mediator of necrosis, in the case of death receptors (Meylan and Tschopp, 2005; Festjens et al, 2007). Necrostatin-1 (Nec-1) can block the kinase activity of RIP1 and inhibit TNF-induced necrosis (Degterev et al, 2008). However, the mechanisms involved in activation of RIP1 and the propagation of necrotic signaling have not been clearly elucidated. It was suggested that RIP1 may be activated in response to metabolic changes involving decreases in levels of ATP and NAD, or by decreases in intracellular pH arising from lactate production during ischemia (Van Herreweghe et al, 2010). *In vivo*, necrosis occurs mainly after ischemia-reperfusion in the heart and brain. Therefore, necrosis inhibitors such as necrostatins could be potential therapeutic tools for treating human diseases that involve ischemia-reperfusion damage, such as cardiac infarction, stroke, traumatic brain injury and organ transplantation.

The results of this study show that HeLa cells treated with higher concentrations of H_2O_2 underwent necrosis whereas, lower concentrations induced apoptotic cell death (Article 1: Figure 9). This switch between apoptosis and necrosis could be explained by two possible mechanisms: 1) the inactivation of caspases due to oxidation/S-nitrosylation of their active site thiol groups by ROS (Samali et al, 1999; Melion et al, 1997; Hampton et al, 1997); and 2) a drop in cellular levels of ATP due to the failure of mitochondrial energy production by oxidants (Leist et al, 1999). Several studies have shown that H_2O_2 induces apoptosis in different cell types (Son et al, 2010; Siu et al, 2009; Takeyama et al, 2002; Blanco et al, 2005; Matsura et al, 1999; Ramachandran et al, 1998), but the mechanisms involved are not completely understood.

Induction of the mitochondrial pathway of apoptosis by H_2O_2

Stimulation of the intrinsic mitochondrial apoptotic pathway by ROS and mitochondrial DNA damage promote outer membrane permeabilization and release of mitochondrial inner membrane proteins into the cytosol, leading to the activation of both caspase-dependent and -independent mitochondrial apoptosis (Ryter et al, 2007). The induction of mitochondrial apoptosis by H_2O_2 is well established in the literature and was reported in different cellular models such as pancreatic beta cells (Sampson et al 2010), human keratinocytes (Wang et al, 2010), neuronal PC12 cells (Crispo et al, 2010; Cai et al, 2008), H9c2 (rat cardiomyocyte) cells (Eguchi et al, 2008), and a human retinal pigment epithelial cell line, ARPE-19 (Kim et al, 2003). This was apparent by a decrease in mitochondrial membrane potential, release of cytochrome c, caspase-9 and -3 activation, PARP cleavage and DNA fragmentation. However, the detailed signaling mechanisms involved are not completely understood.

An important finding from this study is that H_2O_2 caused both caspase-dependent and caspase-independent mitochondrial apoptosis in HeLa cells. Activation of caspase-dependent mitochondrial apoptosis by H_2O_2 was evident by Bax translocation to mitochondria, loss of mitochondrial membrane potential, cytochrome c release into the cytosol, activation of caspase-9 and -3, PARP cleavage and nuclear chromatin condensation. For caspase-independent apoptosis, H_2O_2 caused AIF release from mitochondria into the cytosol, and its translocation to the nucleus after short exposure times (1 h) (Article I: Figure 11A-D). It was reported that AIF, embedded in the mitochondrial inner membrane, has to be cleaved from this membrane in order to become a soluble, apoptotic protein that can be released from mitochondria (Otera et al, 2005). This cleavage can be mediated by the activation of cysteine proteases such as calpain and cathepsins (Yuste et al, 2005). Our observations are consistent with these studies since AIF release and calpain activation both occurred during a 1 h treatment with H_2O_2. Thus, mitochondrial proteins released by H_2O_2 can trigger apoptosis through both caspase-

dependent and caspase-independent mechanisms in HeLa cells. Transfection and gene knockdown using SiRNA directed against AIF and studying H_2O_2-induced apoptosis (Hoechst staining for chromatin condensation) in both knockdown and control cells would provide a strong evidence for the role of AIF in H_2O_2-induced caspase-independent apoptosis. H_2O_2-induced AIF release from mitochondria and its translocation to the nucleus were also reported in other cell lines such as human retinal pigment epithelial (ARPE 19) cells (Ho et al, 2006), mouse differentiated C2C12 myotubes (Siu et al, 2009) and human microglial cells (Braun et al, 2002).

Induction of death receptor pathway of apoptosis by H_2O_2

In the literature, very few studies have shown the induction of apoptosis through death receptors in cells exposed to oxidative stress. Furthermore, the mechanisms are not well defined and are dependent on cell type. Another novel contribution of this study is that the exposure of HeLa cells to H_2O_2 induced Fas-mediated death receptor apoptosis. This was evident by increased expression of the death ligand FasL, translocation of adaptor protein FADD to the plasma membrane and the activation of caspase-8. Once activated, the initiator caspase-8 in turn processed caspase-2. The activation of caspase-2 by oxidative stress is another novel finding of this study. Pre-treatment with a caspase-8 inhibitor reduced the enzymatic activity of caspase-2, confirming the role of caspase-8 in the activation of caspase-2 during oxidative stress. Once procaspases-8 and 2 are activated, they can induce a cross-talk pathway between the death receptor and mitochondrial pathways, through the cleavage of Bid (Youle and Strasser, 2008; Lavrik et al, 2006). H_2O_2 also induced Bid cleavage and t-Bid translocation to mitochondria. Cleavage of Bid and mitochondrial t-bid translocation were completely suppressed by caspase-8 and -2 inhibitors. This suggests that both caspase-8 and caspase-2 can process Bid and contribute to the mitochondrial amplification of H_2O_2-induced cell death by apoptosis.

One of the important strategies to assess the contribution of specific caspases to a given apoptotic signaling pathway is to treat the cells with their respective inhibitors that are commercially available. A number of specific and broad spectrum peptide caspase inhibitors have been developed based upon substrate specificity profiles for the caspases (Berger et al, 2006; Callus and Vaux, 2007). These inhibitors act as pseudosubstrates for caspases. Irreversible binding of these inhibitors to the caspase active sites, results in inhibition of enzyme activity thus preventing apoptosis (Callus and Vaux, 2007). A study determining the specificity of peptide-based inhibitors using a variety of sequences revealed that some of the inhibitors were able to inhibit more than one caspase, with little selectivity (Garcia-Calvo et al, 1998). Problems of specificity have also been reported by other studies (Berger et al, 2006a; Berger et al, 2006b; McStay et al, 2008). For such reasons, using peptide-based inhibitors may lead to flawed conclusions as they may not target a single caspase in an environment where multiple caspases exit. Several other approaches in addition to inhibitors such as, the use of RNA interference, caspase-deficient cells from knockout mice, and variants of cell lines lacking caspases can be used, which provides stronger evidence in determining the role of individual caspases.

In comparison to caspase-8, little is known about the activation and function of caspase-2, which is controversial. The role of caspase-2 in cell death pathways appears to depend on both the apoptotic stimulus and cell type (Bergeron et al, 1998). The activation of caspase-2 has been demonstrated in apoptosis induced by different stresses such as UV irradiation, trophic factor withdrawal, anti-Fas, cytokine deprivation, β-amyloid, etoposide, and other stress stimuli (Zhivotovsky and Orrenius, 2005). Studies show that activation of caspase-2 can occur either by the PIDDosome (Tinel et al, 2007; Tinel and Tschopp, 2004) or the DISC (through caspase-8) (Olsson et al, 2009; Manzl et al, 2009). It was reported that down-regulation of caspase-2 in human leukemic cells significantly prevents Bid cleavage and abolishes cytochrome c release and caspase-3 activation after Fas stimulation (Droin et al, 2001). The involvement of Bid in caspase-2 mediated

cytochrome c release and apoptosis was also shown in mouse embryonic fibroblasts (MEFs) exposed to heat shock (Bonzon et al, 2006). Bid-null MEFs were relatively resistant to apoptosis induced by active caspase-2. Our observations are in agreement with these studies where a caspase-2 inhibitor decreased Bid cleavage, t-Bid translocation to mitochondria and chromatin condensation, confirming its involvement in apoptosis induced by H_2O_2 in HeLa cells. Together, these studies suggest that activation of caspase-2 occurs upstream of mitochondrial outer membrane permeabilization (MOMP). A more recent study shows that cleavage of Bid by caspase-2 also acts as a cross-talk between ER stress and mitochondrial apoptosis, where ER stress inducers caused caspase-2-mediated cleavage of t-Bid, followed by MOMP and cytochrome c release (Upton et al, 2008). Various studies have reported the involvement of caspase-2 in apoptosis induced by chemotherapeutic drugs (Panaretakis et al, 2005; Vakifahmetoglu et al, 2006). Silencing of caspase-2 by siRNA was reported to inhibit cisplatin-induced Bax activation and apoptosis (Cao et al, 2008), while down-regulation of caspase-2 to inhibited etoposide-induced death (Lassus et al, 2002; Robertson et al, 2002). The neurons derived from caspase-2-null mice showed resistance to β-amyloid-mediated death, also suggesting its role in neuronal death (Troy et al, 2000).

Our results also show that H_2O_2-induced death receptor apoptosis was mediated through Fas, since H_2O_2-induced caspase-8, -2 and -9 activation and chromatin condensation were inhibited by the Fas/FasL antagonist (Kp7-6). The inhibition of H_2O_2-induced FasL up-regulation and caspase-8 activation by the antioxidant PEG-catalase confirms the role of H_2O_2 in FasL induction and activation of the death receptor pathway. H_2O_2-induced apoptosis through death receptors was shown in a few other studies, but generally at higher pro-oxidant concentrations and longer exposure times than those in HeLa cells. Up-regulation of FasL and Fas by H_2O_2 was shown in human endothelial cells (HUVEC) and murine intestinal epithelial cells (Denning et al, 2002; Suhara et al, 1998). In human leukemia (HL-60) cells, H_2O_2 activated a caspase-8 mediated signalling

pathway leading to caspase-3 activation (Zhuang et al, 2000). Taken together, the results from these studies indicate that the mechanisms of H_2O_2-induced apoptosis are clearly dependent on cell type.

ROS also play a role in TNF-α and TRAIL-induced apoptosis (Shen et al, 2006; Lee et al, 2002). Exposure of cells to TNF-α caused an increase in intracellular ROS levels. The source of ROS generation was mitochondria and appeared to be mediated by JNK (Shen et al, 2006). Although the mechanism of mitochondrial ROS generation mediated by JNK is not well established, one possible source of intracellular ROS in TNF-α-stimulated cells was considered to be the mitochondrial ETC (Goossens et al, 1999). In a mouse fibrosarcoma cell line (L929), TNF-α induced a caspase-independent, necrosis-like cell death, which was dependent on both ROS and mitochondria (Gossens et al, 1995; Vercammen et al, 1998). Furthermore, antioxidants such as N-acetylcysteine (NAC), SOD and thioredoxin afforded protection against TNF-α-induced cytotoxicity. Scavenging of ROS by antioxidants also abrogated caspase activation and apoptosis induced by TRAIL (Shen et al, 2006). Interactions between ROS and TRAIL-mediated apoptosis have implications for several human disease states. Oxidative stress has been implicated in several pathological conditions such as cancer and neurodegenerative disorders. Damaging effects of ROS, such as protein oxidation and lipid peroxidation, have been reported within specific brain regions in patients with Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Andersen, 2004). Furthermore, β–Amyloid has been shown to produce H_2O_2 in cultured cells (Behl et al, 1994). H_2O_2 sensitized human astroglial cells to TRAIL-induced apoptosis through up-regulation of DR5 expression (Kwon et al, 2008). Several types of tumors are resistant to TRAIL-induced cytotoxicity. In human malignant astrocytoma cells, ligation of the TRAIL receptor led to generation of intracellular ROS through caspase-dependent proteolytic activation of protein kinase C delta (PKCdelta) and subsequent activation of the NADPH oxidase 4 (NOX4) complex (Choi et al, 2010). Caspase-dependent cell death

induced by TRAIL ligation was inhibited by oxidative modification of the active site thiol of caspase-3, leading to resistance to TRAIL-mediated apoptosis.

Role of H_2O_2 in ER stress and ER-mediated apoptosis

The activation of the UPR by mild stress appears to be an adaptive response to maintain cell survival and function. However, more severe or prolonged stress causes cell dysfunction and death, resulting in tissue damage that can contribute to the development and progression of human diseases such as diabetes, atherosclerosis and neurological disorders (Malhotra and Kaufman; 2007).

Unlike the cytosol, the ER has a unique oxidizing environment that promotes protein folding and the formation of disulphide bonds (Gorlach et al, 2006). It was suggested that protein folding and generation of ROS as a by-product of protein oxidation in the ER are closely linked events, since they both cause ER stress-mediated cell death (Malhotra and Kaufman, 2007). ER stress and oxidative stress appear to be closely related events, although the molecular pathways that link these processes are poorly understood.

This study shows that H_2O_2 induced both ER stress and ER-mediated apoptosis in HeLa cells. A short exposure (15 min) of cells to oxidative stress (15-50 μM H_2O_2) led to activation of the UPR, shown by increased expression of p-PERK, p-eIF2α, p-IRE1α, and ATF6 cleavage (Article III: Figure 5, 6, 7, 8). However, longer exposure (1-3 h) to H_2O_2 induced ER-mediated apoptosis, which was evident by increased expression of CHOP, and the activation of calpain, caspase-7, -4, -12 and -3 (Article III: Figure 10, 12, 13, 14, 15). Calcium, calpain and caspase-7 were shown to be upstream factors in the activation of ER-mediated apoptosis by H_2O_2 (Article III: Figure 15). The results of this study provide evidence for the involvement of the ER in oxidative stress-induced apoptosis. H_2O_2-induced activation of the UPR and apoptosis was also reported in other cell lines. In human oral keratinocytes, H_2O_2 increased expression of p-PERK, p-eIF2α,

Bip/GRP78, CHOP, and caused cleavage of caspase-4 (Min et al, 2008). The oxidant tert-butylhydroperoxide caused up-regulation of CHOP and Bip/GRP78 in retinal pigment epithelial cells (He et al, 2008). In hepatocytes, tert-butylhydroperoxide induced activation of caspase-7 and caspase-12, which were implicated in apoptosis through the ER (Haidara et al, 2008). The results of our study demonstrate that H_2O_2-induced both ER stress and ER-mediated apoptosis in HeLa cells. Activation of ER stress (UPR) occurred during earlier time periods i.e. during shorter exposure times (15 min) to H_2O_2 whereas ER-mediated apoptosis was observed during longer exposure times to H_2O_2 (1-3 h). Inhibition of caspase-4 and -12 activity by calcium chelator (BAPTA-AM) and inhibitors of calpain and caspase-7 confirms their roles as upstream initiating factors leading to caspase-4 and -12 activation.

Both ER stress and ROS can stimulate Ca^{2+} release from the ER lumen (Gorlach et al, 2006). The close proximity of the ER and mitochondria leads to mitochondrial calcium accumulation, further increasing mitochondrial ROS production through the ETC, leading to opening of the permeability transition pore and the release of mitochondrial inner membrane proteins (Jacobson et al, 2002). The other important consequence of deregulation of intracellular calcium homeostasis is the activation of nitric oxide synthase, which increases nitric oxide generation. It was reported that nitric oxide can induce apoptosis involving CHOP in microglial cells through the ER stress pathway (Kawahara et al, 2001). In addition, pancreatic islets and peritoneal macrophages from CHOP-knockout mice showed increased resistance to NO-induced apoptosis (Oyadomari et al, 2001; Gotoh et al, 2002). Peroxynitrite, a by product of the reaction between superoxide and nitric oxide, caused ER stress in cultured endothelial cells, resulting in cell dysfunction and apoptosis (Dickhout et al, 2005).

Role of p53 in pathways of apoptosis induced by oxidative stress

Different stress conditions, including ROS and chemical DNA-damaging agents, can up-regulate and (or) activate p53 through phosphorylation. Phosphorylation of p53 at ser15 and ser20 can lead to its stabilization and activation (Liu et al, 2008). Recent studies show that phosphorylation of p53 on Ser46 following DNA damage is important for preferential transactivation of proapoptotic genes (Kodama et al, 2010; Saito et al, 2002). Activated p53 protein can induce cellular pathways leading to cell cycle arrest in the G1 phase, or to apoptosis dependent on the cell type and injury, via differential activation of target genes (Yoshida and Miki 2010; Liu et al, 2008). At the transcriptional level, phosphorylated p53 induces transcription of pro-apoptotic proteins such as Bax, Bid, Puma, Noxa, Apaf-1 and FasL, and it suppresses the activity of anti-apoptotic proteins such as Bcl-2 and IAPs (Yoshida and Miki, 2010; Yu and Zhang, 2008; Muller et al, 1998). Several recent observations also suggest a role for p53 in caspase-2 activation (Zhivotovsky and Orrenius, 2005). Activation of caspase-2 can in turn induce the mitochondria-mediated apoptotic pathway. A new finding from this study is that, in HeLa cells, induction of apoptosis by H_2O_2 was mediated by p53 as an upstream factor. This study clearly demonstrates that H_2O_2 causes phosphorylation of p53 at Ser15 and Ser46, leading to its activation. The activation, of p53 was further confirmed by inhibition of the expression of p53 target proteins, FasL and PUMA, and Bax translocation to mitochondria by the p53 inhibitor, pifithrin-α. H_2O_2-induced activation of caspase-8, -2, -9 and -3, and chromatin condensation was also decreased by p53 inhibitor pifithrin-α. Furthermore, the findings using pifithrin-α indicate that p53 is an up-stream factor leading to the activation of death receptor and mitochondria-mediated apoptosis in H_2O_2-treated HeLa cells. Rat neural AF5 cells and glioma cells treated with H_2O_2 also showed increased protein expression of p53, phosphorylated p53 at Ser15, Puma, Noxa and Bax (McNeill-Blue et al, 2006; Datta et al, 2002). p53 expression was also shown to be induced during apoptosis mediated by nitric oxide (Messmer et al, 1994).

The chemical inhibitor of p53, pifithrin was isolated for its ability to block p53-dependent transcriptional activation (Komarov et al, 1999). Studies show that pifithrin suppress transactivation of p53-responsive genes encoding p21, Mdm2, cyclin G, and Bax (Komarov et al, 1999; Tamagno et al, 2003; Nakaso et al, 2004; Duan et al, 2002; Dagher, 2004; Kelly et al, 2003). In vitro and In vivo studies also show that pifithrin protect different cell types against p53-dependent apoptosis induced by a multitude of stimuli (Camphausen et al, 2003; Culmsee et al, 2001; Schafer et al, 2003; Leker et al, 2004; Kuo et al, 2004; Walton et al, 2005; Fraser et al, 2006). Antiapoptotic effect of pifithrin was found to be p53-dependent and involved suppression of caspase activation. In neuronal cells, caspase inhibition by pifithrin was associated with suppression of mitochondrial dysfunction (Nakaso et al, 2004; Duan et al, 2002). A direct inhibition of p53 translocation to mitochondria was observed in kidney cells as a result of pifithrin action (Dagher, 2004; Kelly et al, 2003). DNA damage studies show that the levels of nuclear but not those of cytoplasmic p53 protein, were found to be lowered by pifithrin (Komarov et al, 1999; Lorenzo et al, 2002; Liu et al, 2004; Dagher, 2004; Kelly et al, 2003; Schafer et al, 2003) suggesting that pifithrin might modulate the nuclear import or export (or both) of p53 or decrease stability of nuclear p53. Besides p53 dependent effects, pifithrin can also suppress heat shock and glucocorticoid receptor signaling (Komarova et al, 2003). Recent studies do show that pifithrin, can also target other proteins such as the aryl hydrocarbon receptor (AhR) (Hoagland et al, 2005), ICAM-1 (Gorgoulis et al, 2003), androgen receptor (Cronauer et al, 2004), and CD95 (Lorenzo et al, 2002). These studies suggest that pifithrin effects are not limited to p53 and downstream components of p53 pathway but involve other cellular factors and signal transduction pathways. It is, therefore, extremely important to include controls of p53 specificity for accurate interpretation of experimental results. Alternatively other types of inhibitors such as gene knockouts or knockdowns using siRNA would be useful for stronger conclusions.

Interactions between p53, ROS and antioxidants are complex and not well understood (Liu et al, 2008). During apoptosis induced by tumor promoter 12-O tetradecanoylphorbol-13-aectate, p53 underwent translocation to mitochondria, where it directly bound and inhibited MnSOD (Zhao et al, 2005). p53-mediated MnSOD inactivation led to subsequent activation of p53 transcriptional activity and induction of pro-apoptotic target genes such as Bax. Translocation of p53 to the nucleus and induction of Bax protein were blocked by a SOD mimetic (MnTE-2-PyP^{5+}). Over-expression of catalase protected HepG2 cells against apoptosis induced by DNA-damaging agents (Bai and Cederbaum, 2003). The protective effect was associated with decreased p53 phosphorylation and accelerated proteasomal degradation of p53 protein. p53 is a redox-sensitive protein, which undergoes oxidation at cysteine residues that contain redox-sensitive thiol groups (Liu et al, 2008). During oxidative stress, p53 undergoes S-glutathionylation, which inhibits DNA binding activity of p53. This process was reversed when GSH levels were increased by the antioxidant NAC.

Studies show that p53 target gene Tp53-induced glycolysis and apoptosis regulator (TIGAR) regulates intracellular ROS levels by modulation of the glycolytic pathway (Bensaad et al, 2006). By decreasing the glycolytic rate and redirecting glycolytic intermediates to the oxidative branch of the pentose phosphate pathway, TIGAR caused an increase in NADPH production, which allows for scavenging of intracellular ROS by reduced glutathione. Thus, TIGAR reduces the sensitivity of cells to p53-dependent apoptosis induced by oxidative stress (Bensaad et al, 2006).

H_2O_2 and Cancer

Oxidative deamination of polyamines by amine oxidases results in the generation of H_2O_2 and aldehydes such as acrolein (Toninello et al, 2006; Agostinelli et al, 2004; Averill-Bates et al, 1994; Lord-Fontaine et al, 2001). The naturally occurring polyamines such as putrescine, spermidine and spermine play an important role in cellular growth and

differentiation (Wallace et al, 2003). Tumour cells express high levels of polyamines and depletion of polyamines leads to inhibition of tumour growth (Heby and Persson, 1990; Bachrach, 2004). The oxidation products such as H_2O_2 and acrolein were shown to be responsible for cytotoxicity and caspase-mediated apoptosis induced by amine oxidase and spermine in mouse melanoma cells (Averill-Bates et al, 2008). To take advantage of higher levels of polyamines in tumor versus normal tissues, toxic products such as H_2O_2 and acrolein could be generated in situ, by delivering amine oxidases into tumors to induce cytotoxicity (Averill-Bates et al, 2005). Thus, generation of toxic products such as H_2O_2 and acrolein by amine oxidases could be a promising new tool in cancer treatment. The results of our study demonstrate the signalling mechanisms implicated in H_2O_2–induced apoptosis.

Mild heat-pre-conditioning causes resistance of cells to apoptosis induced by oxidative stress

Oxidative stress plays a major role in various pathological conditions such as cancer, diabetes, cardiovascular disease, Parkinson's and Alzheimer's disease (neurological disorders), ischemia-reperfusion and ageing (reviewed by Valko et al, 2007). The results of this study show that mild heat (40°C) pre-conditioning protects cells against oxidative stress-induced apoptosis. An important new result is that mild thermotolerance increased the expression of major antioxidants (MnSOD, catalase, glutathione and γ-GCS) as well as ER stress proteins (p-PERK, p-eIF2α). It is well established that mild thermotolerance induces the expression of Hsps (Przybytkowski et al, 1986; Bettaieb and Averill-Bates, 2005). All of the events in the mitochondrial, death receptor and ER-mediated pathways of apoptosis were found to be diminished in thermotolerant cells. These protective effects could be attributed to the ROS scavengers, ER stress proteins and Hsps (discussed in articles 1, 2 and 3). Hsps (Beere, 2004) and ROS scavengers are known to have anti-apoptotic properties (Portt et al, 2011). Mild thermotolerance even rendered cells resistant to H_2O_2-induced cytotoxicity (confirmed by clonogenic cell survival assay). The

results from this study suggest that mild heat pre-conditioning at 40°C plays a cytoprotective role by up-regulating anti-apoptotic genes.

Hsps are expressed at increased levels in a wide range of tumors (Calderwood et al, 2006). Other anti-apoptotic molecules that are up-regulated in tumors include ROS scavengers (reviewed in Portt et al, 2011). Mild thermotolerant HeLa cells with increased expression of Hsps and ROS scavengers are resistant to H_2O_2-induced apoptosis. This suggests that tumors that over-express anti-apoptotic molecules could exhibit resistance to therapeutic agents, such as radiation and oxidant-generating drugs, which kill cancer cells via oxidative stress.

$O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ are produced during ischemia-reperfusion through metabolism of xanthine and hypoxanthine by xanthine oxidase (Szocs et al, 2004). The massive production of ROS during ischemia/reperfusion leads to tissue injury and death, thus causing serious complications in organ transplantation, stroke and myocardial infarction (Kasparova et al, 2005). Ischemic pre-conditioning (IPC) and post-conditioning (IPOC) have been shown to protect the heart against ischemia-reperfusion (I/R) injury (reviewed by Portt et al, 2011; Murry et al, 1986; Zhao et al, 2003). Activation of kinases such as ERK and Akt (Portt et al, 2011), Hsps (Yellon and Latchman, 1992) and antioxidants (Hoshida et al, 1993) were found to be involved in the cardio-protective effects of ischemic pre- and post-conditioning. Over-expression of anti-apoptotic protein IAP-2 in transgenic mice was shown to render the heart more resistant to apoptosis and I/R injury (Chua et al, 2007). The pre-conditioning with ER stress-inducing conditions such as ischemia or tunicamycin protected cultured neurons or cardiomyocytes from subsequent oxidative stress and I/R injury (Hayashi et al, 2003; Zhang et al, 2004; Liu et al, 2008).

Antioxidants are able to protect cells against the nocive effects of several environmental toxins. Mitochondrial SOD was shown to protect *Saccharomyces*

cerevisiae cells against stresses such as high osmolarity, heat and metalloid stress (Dziadkowiec et al, 2007). The protective role of NAC against lead-induced cytotoxicity and oxidative stress was reported in human liver carcinoma (HepG2) cells (Yedjou and Tchounwou, 2007). Recent studies also demonstrated protective roles of vitamin E against nickel- and/or chromium-induced toxicity (Rao et al, 2009) and ascorbic acid (vitamin C) against lead toxicity (Bhattacharjee et al, 2003) in mouse ovary and erythrocytes. A protective role of glutathione and metallothioneins against cadmium toxicity was reported in Chinese hamster cells (Ochi et al, 1988; Park et al, 2001). Studies showed that over-expression of antioxidants such as SOD or glutathione peroxidase protected against the combined paraquat and maneb-induced Parkinson disease phenotype (Thiruchalvam et al, 2005).

Together, these studies indicate that increased expression of anti-apoptotic genes (ROS scavengers and Hsps) could serve to delay or prevent cell death in response to different stress stimuli. Pre-conditioning-mediated stimulation of endogenous anti-apoptosis proteins might have therapeutic potential in limiting apoptosis in neurological diseases such as Parkinson's, Alzheimer's or stroke (Portt et al, 2011), and against toxicity of environmental pollutants.

FUTURE PERSPECTIVES

Different stress conditions such as hypoxia, nutrient starvation, hormonal imbalance and oxidative stress can also induce autophagy. Autophagy is rapidly activated as an adaptive catabolic process in response to the above metabolic stresses, whereby cells eliminate damaged cytoplasmic components (mitochondria, ER). The cytoplasmic contents are sequestered within double-membrane vacuoles (autophagosomes) and then delivered to lysosomes for degradation (Kroemer et al, 2010; Portt et al, 2011). Degradation of cytoplasmic contents generates free fatty acids and amino acids that can

be recycled and used for de novo synthesis of proteins and in maintaining mitochondrial energy (ATP) production and thereby promote cell survival (Levine and Kroemer, 2009). Under most circumstances, autophagy constitutes a stress adaption pathway that promotes cell survival during nutrient limitation (starvation) or acts as a defence mechanism against different environmental stresses.

Under conditions of prolonged stress, autophagy may also serve as an alternative form of regulated non-apoptotic (type II) cell death (Portt et al, 2011). The autophagy related genes (atg), Beclin-1 and the mammalian target of rapamycin (m-TOR) signalling pathway mediate autophagy. Autophagy is also linked to apoptotic cell death, indicating the existence of a 'molecular switch' between these two processes. Studies report that apoptosis and autophagy may be interconnected and even regulated simultaneously by the same trigger (Liu et al, 2009; Cheng et al, 2009). ROS and Ca^{2+} may provide a common link between cellular stress signals and the initiation of autophagy, since ROS (specifically, H_2O_2) accumulation has been reported to result in inactivation of the cysteine protease ATG4. This leads to accumulation of the ATG8-phosphoethanolamine, which initiates autophagosome formation (Scherz-Shouval et al, 2007). Ca^{2+}-stimulated calpain activation contributes to induction of both autophagy (Demarchi et al, 2006) and apoptosis (Yousefi et al, 2006). Calpain-mediated cleavage of Atg5, to a 24kDa fragment, switches autophagy to apoptosis, where the truncated fragment translocates from the cytosol to mitochondria, mediating cytochrome c release and caspase activation (Yousefi et al, 2006). ER stress has also been shown to act as a mediator connecting apoptosis and autophagy. Activation of the PERK-eIF2α or IRE1-JNK pathways promotes autophagy through facilitating or stimulating light chain 3 (LC3) conversions and autophagosome formation (Moretti et al, 2007). In addition, studies show that p53 can transactivate damage regulated autophagy modulator (DRAM), a lysosomal protein that can stimulate the accumulation of autophagic vacuoles during autophagy (Crighton et al, 2006).

The above studies show that regulators such as ROS, calpain, ER stress and p53, which play a major role in apoptosis induction, can also induce autophagy. As a future perspective, it would be interesting to determine the role of ROS (H_2O_2) in the induction of autophagic cell death. The results of the present study show that the exposure of cells to a mild H_2O_2 stress activates the ER stress pathway as an adaptive survival response (article III). We can therefore hypothesize that H_2O_2 could also induce autophagy as an adaptive survival mechanism at lower doses and trigger cell death by autophagy and/or apoptosis at higher doses. It would also be interesting to understand the molecular links between autophagy and apoptosis that should improve our knowledge about cell death pathways, which in turn may lead to the development of new strategies for therapeutic induction or inhibition of cell death. Targeting autophagic signalling pathways could be an alternative way of inducing cell death in cancer cells that show resistance to apoptosis. However, in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, autophagy could play a cyto-protective role by maintaining neuronal homeostasis and protein quality control.

In conclusion, improved understanding of mechanisms of adaption to stressful and extreme environments provides the basis for understanding environmental health problems, performing toxicological risk assessment and in clinical applications against many disease states.

APPENDIX A

ACROLEIN INDUCES A CELLULAR STRESS RESPONSE AND TRIGGERS MITOCHONDRIAL APOPTOSIS IN A549 CELLS.

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Acrolein induces a cellular stress response and triggers mitochondrial apoptosis in A549 cells

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ABSTRACT

Acrolein is a highly reactive, α,β-unsaturated aldehyde that is an omnipresent environmental pollutant. Humans are exposed to acrolein in food, vapors of overheated cooking oil, cigarette smoke and by combustion of organic products. Acrolein is a toxic by-product of lipid peroxidation resulting from oxidative stress, which is implicated in pulmonary, cardiac and neurodegenerative diseases. Low dose exposure to toxic compounds often leads to adaptive responses. If the adaptive response does not counteract the adverse exposure, death processes such as apoptosis will eliminate the cell. This study investigates the activation of antiapoptosis survival factors in relation to the induction of cell death by apoptosis, following exposure to low doses of acrolein, in A549 human lung cells. Exposure to acrolein (<15 μM, 30 min) activated the survival factor AKT, which led to phosphorylation of Bad and induction of antiapoptosis proteins cIAP1/2. Acrolein (10–50 μM, 30–60 min) increased reactive oxygen species and caused mitochondrial membrane hyperpolarisation. Inhibition by the antioxidants catalase, polyethylene glycol-catalase, sodium pyruvate and MnTBAP showed that acrolein-induced reactive oxygen species were responsible for mitochondrial membrane hyperpolarisation. Acrolein (3–27 μM, 30–60 min) activated early stage processes in the mitochondrial pathway of apoptosis, such as Bax translocation to mitochondria, cytochrome c release, caspase-9 activation, and translocation of apoptosis-inducing factor to the nucleus. Acrolein (10–50 μM) triggered later stage processes such as activation of caspases-3, -7 and -6, phosphatidylserine externalization and cleavage of poly(ADP)ribose polymerase after longer times (2 h). These events were inhibited by polyethylene glycol-catalase, showing that apoptosis was mediated by overproduction of reactive oxygen species by acrolein. The novel findings show that antiapoptosis processes dominate at low dose (<15 μM)/shorter exposure times to acrolein, whereas proapoptotic processes dominate at higher dose (10–50 μM)/longer exposure times. Acrolein induced apoptosis through the mitochondrial pathway that was mediated by reactive oxygen species.

Abbreviations: AIF, apoptosis-inducing factor; AKT, protein kinase B; Apaf-1, apoptosis protease activating factor-1; Bax, Bcl-associated X protein; Bcl-2, B-cells lymphoma 2; Bcl-XL, Bcl-associated X long protein; Bid, BH3-interacting domain death agonist; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; cIAP, cellular inhibitors of apoptosis; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's modification of Eagle's medium; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; Fas, fibroblast-associated receptor; FasL, Fas ligand; FACS, fluorescence activated cell sorter; FBS, fetal bovine serum; FCCP, *p*-trifluoromethoxy-phenyl-hydrazone; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; αMEM, minimum essential medium alpha; MnTBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; MOPS, 3-(N-morpholino)-propane sulfonic acid; NADH, nicotinamide adenine dinucleotide; PARP, poly(ADP-ribose) polymerase; PEG, polyethylene glycol; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene difluoride; Rho123, rhodamine 123; RFI, relative fluorescence intensity; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; tBid, truncated Bid.

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1. Introduction

Acrolein is an α,β-unsaturated aldehyde, which is an ubiquitous environmental pollutant [1]. It is generated as a product of the incomplete combustion during forest and house fires, combustion of plastics, cooking of food and motor vehicle emissions. A major source of acrolein is cigarette smoke, which generates 50–90 ppm of acrolein per cigarette. Acrolein is mainly utilized as a biocide, as well as an intermediate in the production of acrylic acid and DL-methionine, and as a starting material for acrylate polymers and numerous other agents [2]. Acrolein is also produced *in situ*, during the normal intermediary catabolism of various amino acids and polyamines [3], and as a metabolite of allylic alcohol or the anticancer drug cyclophosphamide [4]. An important source of acrolein is as a by-product of cellular lipid peroxidation associated with oxidative stress [2].

Acrolein is a highly reactive compound with an electrophilic nature [5], which permits it to bind and deplete cellular nucle-

ophiles. Acrolein causes rapid depletion of the antioxidant glutathione and is able to initiate lipid peroxidation [2]. Furthermore, acrolein binds to nucleophilic sites in DNA and forms DNA adducts [2]. It is mutagenic and genotoxic to human cells [2], and was shown to decrease the proliferation of human lung carcinoma cell lines [6]. Acrolein can also form protein adducts and causes severe conformational changes in proteins [7]. Given that inhalation is the major route of exposure, acrolein has been associated with many lung diseases such as asthma [8], chronic obstructive pulmonary disease (COPD) [9], which often includes chronic bronchitis and emphysema [10], cystic fibrosis [8] and lung carcinogenesis [11]. However, the biochemical pathways involved in acrolein-induced cellular responses and toxicity are not entirely understood.

Low dose chronic exposure to toxic compounds can often lead to adaptive responses [12]. Adaptive responses allow cells and organisms to continue normal function in the face of an adverse stimulus. These responses often involve changes in gene and protein expression, including the induction of defenses to enable the cell to survive [13]. Organisms have developed an elaborate system of defense molecules (e.g. antioxidants, heat shock proteins (HSP), antiapoptosis proteins (IAPs) and detoxifying enzymes) and survival signaling pathways (e.g. phosphatidylinositol 3-kinase (PI3K)/AKT, Ras/mitogen-activated protein kinase) to counteract various toxic and environmental stresses. If the adaptive response does not counteract the adverse exposure, then the cell will be eliminated by death processes such as apoptosis [13].

Apoptosis is essential for normal development and maintenance of tissues, but also plays a role in the elimination of damaged cells. It has distinct morphological and biochemical alterations such as DNA fragmentation into 180–200 pb, phosphatidylserine externalization and cellular fragmentation into apoptotic bodies [14]. The mitochondrial pathway of apoptosis is activated by various developmental cues or cytotoxic insults, such as DNA damage, oxidative stress, heat shock and exposure to a variety of toxic compounds. This pathway is strictly controlled by the Bcl-2 family of proteins [15]. Activation of proapoptotic members of this family, such as Bax or truncated Bid (tBid), induces permeabilization of the outer mitochondrial membrane, which releases soluble proteins from the intermembrane space into the cytosol, where they promote caspase activation and cell death [16]. The best studied of these proteins is cytochrome c, which binds to apoptosis protease activating factor-1 (Apaf-1) and leads to the assembly of an apoptosome complex. The apoptosome can bind procaspase-9 and cause its auto-activation through a conformational change [14,16]. Upon activation, caspase-9 propagates the caspase cascade through activation of effector caspases-3 and -7 [16]. Caspase-3 in turn activates caspase-6 and can also participate in a feedback amplification loop to further process caspase-9 [17,18]. Thus, activated caspase-9 can rapidly amplify the death signal. Furthermore, proteins, such as endonuclease G and apoptosis-inducing factor (AIF), which normally reside in the mitochondria, are also released during apoptosis. These proteins translocate from mitochondria to the nucleus, where they induce DNA fragmentation in a caspase-independent manner [18].

This study investigates whether exposure to low doses of acrolein (<50 μM) can induce antiapoptosis cell survival factors such as AKT and IAPs, in relation to the induction of cell death by apoptosis through the mitochondrial pathway. Since one of the major routes of exposure to acrolein is the lung via inhalation, the A549 cell line was chosen for this study due to its human pulmonary origin. Improving our understanding of acrolein-induced cellular responses is important given the widespread chronic exposure of humans to low doses of this toxic agent and its potential adverse effects on human health.

2. Materials and methods

2.1. Cell culture

A549 human lung cells (ATCC #CCL-185) were grown in monolayer in Dulbecco's modified Eagle's medium (Invitrogen Canada, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS) (Invitrogen Canada), 2 mM L-glutamine, 3.7 g/l sodium bicarbonate, 1.0 mM sodium pyruvate and 1% penicillin (50 U/ml)–streptomycin (50 μg/ml) (Flow Laboratories, Mississauga, ON, Canada), in tissue culture flasks (Sarstedt, St. Laurent, QC, Canada). The cells were grown to near confluence and then harvested using 0.25% (w/v) trypsin–0.02% (w/v) EDTA solution, washed by centrifugation (1000 × *g*, 3 min) and resuspended in minimum essential medium alpha (αMEM) plus 10% FBS for experimental studies [19,20].

2.2. Treatment with acrolein

Cells (10^6/ml) were incubated with acrolein (Sigma–Aldrich Canada, ON, Canada) (1–1000 μM) for different times ranging from 30 min to 24 h at 37 °C. Cells were pretreated for 1 h with 25 μM of the PI3K/AKT inhibitors LY294002 or 50 nM Wortmannin, 10 μM AKT inhibitor I, 50 μM Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), 10 μM of the general caspase inhibitor Z-VAD (Calbiochem, La Jolla, CA, USA), for 3 h with 300 U/ml of the antioxidant polyethylene glycol-catalase (PEG-catalase) or 2.4 mM PEG MW 5000 (equivalent amount of PEG in PEG-catalase (300 U/ml)), and for 5 min with 1 mM sodium pyruvate or 300 U/ml catalase (Sigma–Aldrich, Canada), where indicated. Cells were washed by centrifugation (1000 × *g*, 3 min) to remove acrolein, and then analysed for apoptotic signaling.

2.3. Catalase activity

Following incubation with 300 U/ml PEG-catalase or 2.4 mM PEG for 3 h, A549 cells (5×10^6) were suspended in 500 μl of PBS. The cell suspension was then sonicated twice for 20 s each (Vibra Cell, Sonics & Materials, Inc., Danbury, CT) and centrifuged (12 000 × *g*, 15 min) to obtain a clear extract [21]. Catalase activity was determined at 25 °C according to Claiborne [22]. The decomposition of hydrogen peroxide by catalase was followed by ultraviolet spectroscopy at 240 nm. The dosage was performed using a solution of 19 mM hydrogen peroxide containing the clear cellular extract in a final volume of 3 ml at 25 °C. One unit was defined as μmol of peroxide consumed/min/10^6 cells.

2.4. Clonogenic cell survival

A549 cells (10^5/ml) were pretreated with or without PEG-catalase (300 U/ml) or 2.4 mM PEG for 3 h, or DMSO or Z-VAD (10 μM) for 1 h, and then exposed to acrolein (0–1000 μM) for 1 h at 37 °C in a final volume of 1.0 ml, in α-MEM containing 10% FBS. After the appropriate time, the cells were washed three times by centrifugation (1000 × *g*, 2 min) to stop the incubation [21]. The cells were resuspended in culture medium, diluted to the appropriate concentration and plated in tissue culture dishes (60 mm × 15 mm), which were incubated at 37 °C in an atmosphere of 5% CO_2 for 10 days. The dishes were then washed with PBS, fixed with 95% ethanol and stained with methylene blue before counting macroscopic colonies (>50 cells). Cytotoxicity was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells were seeded in the control plates, but where there was a loss of cell survival, cells were plated at several different densities to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have previously demonstrated that, in this system, there is linearity between

the number of cells plated and colonies formed over the range of $10–10^4$ [23].

2.5. Flow cytometry analysis of mitochondrial membrane potential

A549 cells (1×10^6) were pretreated with or without 1 mM sodium pyruvate for 5 min, 50 μM MnTBAP for 1 h, 300 U/ml catalase for 5 min, 300 U/ml PEG-catalase or 2.4 mM PEG for 3 h, and then exposed to acrolein or to 5 μM *p*-trifluoromethoxy-phenylhydrazone (FCCP; positive control), in a final volume of 1.0 ml at 37 °C. Then, 2 μM rhodamine 123 (Rho123) was added for 30 min at 37 °C in the dark. Changes in fluorescence (mitochondrial membrane potential) on the FL-1 detector in 10 000 cells were measured by flow cytometry using a FACScan equipped with an argon laser (488 nm) and analysed using Lysis II software (Becton Dickinson, Oxford, UK) [24].

2.6. ROS generation

The generation of pro-oxidants was measured as described by Denning et al. [25], with modifications. A549 cells were pretreated with or without 300 U/ml PEG-catalase for 3 h and then exposed to acrolein. As a positive control, cells were treated with 1 mM hydrogen peroxide (H_2O_2). Then, 10 μM 2′7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) was added. Levels of pro-oxidants in 10 000 cells were determined by flow cytometry using the FL-1 detector.

2.7. Preparation of whole cell lysates

Cells were pretreated with or without 25 μM of LY294002 for 1 h, 50 nM Wortmannin, 10 μM AKT inhibitor I for 30 min, 300 U/ml PEG-catalase or 2.4 mM PEG for 3 h and then exposed to acrolein. Cells were then washed by centrifugation (1000 × *g*, 3 min) in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) [26]. The supernatant was discarded, pelleted cells were resuspended in lysis buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, 25 μM calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and incubated on ice for 1 h. Then, by a 10 min centrifugation step at 2500 × *g* to remove nuclei and unbroken cells, the proteins of whole cell lysates were isolated in the supernatant for the immunodetection of AKT, phospho-AKT (ser473), Bad, phospho-Bad (ser136), cIAP1/2 and PARP.

2.8. Preparation of subcellular fractions

Cells were treated with acrolein and then subcellular fractions were prepared as previously described [26]. Cells were washed in buffer A and resuspended in buffer B containing 0.1 mM dithiothreitol (DTT). Following a 30 min incubation on ice, lysates were homogenised using a hand potter (Kontes glass CO, Duall 22, Fisher, QC, Canada) and centrifuged at 2500 × *g* for 10 min. The supernatant was centrifuged at 2500 × *g* for 10 min to isolate the nuclear fraction in the pellet, while the supernatant was used to isolate mitochondria and cytosol (see below). The nuclear fraction was purified by resuspending the pellet in an equal volume of a buffer containing 20 mM HEPES-KOH (pH 7.9) at 4 °C, 25% glycerol, 420 mM NaCl, 0.2 mM PMSF, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 1% cocktail of protease inhibitors [27]. Samples were incubated on ice for 20 min and the nuclear fraction was isolated from the cellular debris by centrifugation at 15 000 × *g*, for 5 min. This fraction was used for detection of AIF in the nucleus.

The supernatant from the 2500 × *g* centrifugation step was centrifuged at 15 000 × *g*, 15 min. The subsequent pellet was washed with buffer A and centrifuged at 15 000 × *g* for 15 min. The final pellet containing the purified mitochondrial fraction was then resuspended in lysis buffer B for the detection of Bax, cytochrome *c* and AIF. The supernatant was then centrifuged at 100 000 × *g* for 1 h. The resulting supernatant was designated as the cytosolic fraction, which was used for the detection of cytochrome *c* and AIF.

2.9. Immunodetection

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (10%: PARP, cIAP1/2, AIF, AKT, phospho-AKT (ser473); 15%: cytochrome *c*, Bax, Bad, phospho-Bad (ser136)) of cellular proteins was carried out according to Laemmli [28]. Proteins (40 μg) were quantified according to Bradford [29] and then solubilized in Laemmli sample buffer. Electrophoresis was carried out at a constant voltage of 125 V. Cellular proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Milli-pore, Bedford, MA, USA) [30]. The blots were probed with the primary antibodies: anti-PARP, anti-cIAP1/2, anti-AIF, anti-cytochrome *c*, anti-Bax, anti-Bad, anti-phospho-Bad (ser136), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-AKT, anti-phospho-AKT (ser473) (Stressgen, San Diego, CA, USA) in Tris-buffered saline (50 mM Tris base, 150 mM NaCl, 0.1% Tween-20) (TBS-T) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Biosource, Camarillo, CA, USA) diluted in TBS-T containing 5% milk powder. Proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA, USA). Protein expression was analysed using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA), relative to GAPDH.

2.10. Flow cytometry analysis of intracellular expression of Bax, Bcl-2 and survivin

Cells were treated with acrolein and then fixed in 1.0 ml of freshly made cold 0.25% paraformaldehyde for 1 h on ice. After washing, cells were permeabilized in 1 ml of PBS–1% FBS–0.05% Tween-20 for 15 min at 37 °C. Cells were then labeled with anti-Bax, anti-Bcl-2 or anti-survivin monoclonal antibodies (5 μl per 10^6 cells) (Santa Cruz Biotechnology) for 30 min at room temperature. After washing, labeled cells were incubated for 30 min at room temperature in the dark with a secondary FITC-labeled anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Cells (10 000) were analysed by flow cytometry on the FL-1 detector. The specificity of the secondary antibodies was tested by labeling cells, with or without acrolein treatment, with just the secondary fluorescent antibodies without primary antibody [31,32].

2.11. Determination of caspase activity by fluorescence spectroscopy

The activity of caspases was measured as previously described by Hampton and Orrenius [33], with minor modifications. Cells were pretreated with or without 300 U/ml PEG-catalase for 3 h and then exposed to acrolein. After incubation, cells were washed, resuspended and lysed at −80 °C for 30 min. The kinetic reaction was followed for 30 min after addition of the appropriate caspase substrates at 37 °C [30] using a Spectra Max Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, CA, USA). Caspase-9 activity was measured by cleavage of the fluorogenic peptide substrate Ac-Leu-Glu-His-Asp-AFC. For caspase-3, the substrate was Ac-Asp-Glu-Val-Asp-AMC, MCA-Val-Asp-Gln-Val-Gly-Trp-Lys-(DNP)-NH_2 was the substrate for caspase-7 and for caspase-6, the substrate was Ac-Val-Glu-Ile-Asp-AMC (Calbiochem).

2.12. Cell cycle analysis

Following treatment with acrolein, cells were washed, resuspended in 1 ml of cold PBS–1% FBS and then 3 ml of cold 100% ethanol were added drop by drop with agitation. Cells were fixed at −20 °C for 1 h. Cells were resuspended in 500 μl of cell cycle solution (0.05 mg/ml of propidium iodide (PI) and 0.5 μg/ml of RNase A in PBS) and incubated at 4 °C for 24 h in the dark [34]. Cells (10000) were analysed by flow cytometry on the FL-2, FL2-A and FL2-W detectors and analysed using ModFit software (Verity Software House, Topsham, ME, USA) to determine the percentage of cells in apoptosis, G0/G1 phase, S phase and G2/M phase.

2.13. Determination of phosphatidylserine (PS) externalization by Annexin V-FITC staining

A549 cells (10^6/ml) were pretreated with or without PEG-catalase (300 U/ml) or PEG (2.4 mM) for 3 h, or Z-VAD (10 μM) for 1 h, and then exposed to acrolein (0–14 μM) for 2 h. Cells were washed with PBS and resuspended in 1 ml of binding buffer (10 mM HEPES/NaOH, pH 7.5, 140 mM NaCl, and 2.5 mM $CaCl_2$). A volume of 500 μl of cell suspension was incubated with 5 μl of Annexin V-FITC (BD Biosciences Canada, Mississauga, ON, Canada) and 10 μl of propidium iodide (PI) for 10 min at room temperature in the dark [35]. Cells (10000) were then analysed by flow cytometry [36]. Annexin V-FITC fluorescence was detected on the FL-1 detector and PI fluorescence on the FL-3 detector. Four populations of cells were analysed: live control cells (Annexin V^-/PI^-); early stage apoptotic cells (Annexin V^+/PI^-); late stage apoptotic cells (Annexin V^+/PI^+); necrotic cells (Annexin V^-/PI^+). The results are reported as the fraction of total apoptotic cells (early and late stage apoptosis) and necrotic cells.

2.14. Statistics

Data represent means ± SEM from at least three independent experiments performed in duplicate. When not shown, error bars lie within symbols. Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means. The Bonferroni–Holmes adjustment was used to control for the family-wise error rate at a desired level ($\alpha = 5\%$). Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). For significant differences, $(*)P < 0.05$, $(**)P < 0.01$ or $(***)P < 0.001$ for acrolein-treatment compared with control (0 μM); $(0)P < 0.05$, $(00)P < 0.01$ or $(000)P < 0.001$ for cells with or without treatment with sodium pyruvate, MnTBAP, catalase, PEG-catalase, PEG, Z-VAD, Wortmannin, AKT inhibitor I or LY294002, at the same concentration of acrolein.

3. Results

3.1. Activation of survival factors and antiapoptosis proteins by acrolein in A549 lung cells

Exposure to toxic compounds, under less severe stress conditions, can often induce cellular defenses to enable the cell to survive. Therefore, we will determine whether acrolein can activate survival factors such as AKT (protein kinase B) and Bad, and antiapoptosis proteins such as c-IAP and survivin.

3.1.1. Acrolein activated AKT

We began investigating whether acrolein could activate the signaling pathways involved in cell survival. AKT is generally involved in cell survival signaling pathways and is activated in response to many different growth factors, hormones, and external stresses such as heat shock and osmolarity [37]. We evaluated the capacity of acrolein to regulate the expression of AKT and its phosphorylation in A549 cells (Fig. 1A and B). Indeed, acrolein (3–14 μM) caused a significant increase in the total cellular expression of both AKT and phospho-AKT (ser473), after a short exposure time of 30 min, in relation to untreated cells.

3.1.2. Acrolein-induced phosphorylation of Bad

The proapoptotic Bcl-2 family protein Bad regulates apoptosis upstream of mitochondrial-mediated caspase-9 activation. Activation of AKT by phosphorylation plays a survival role by preventing the proapoptotic action of Bad through its phosphorylation at $Ser^{136}P$ [38]. We therefore determined the ability of acrolein to modulate the expression of Bad and its phosphorylation in A549 cells (Fig. 1C–G). Exposure to acrolein (3–14 μM) for 30 min caused a significant increase (58–77%) in total cellular expression of phospho-Bad (ser136). There was also a small increase in total cellular expression of Bad in cells treated with 14 μM acrolein. Acrolein-induced phosphorylation of Bad was inhibited significantly by the PI3K/AKT inhibitor LY294002 (Fig. 1C–E), which suggests that this response could be mediated by PI3K and AKT. However, LY294002 is known to bind reversibly to PI3K, but it can also inhibit the kinase activity of mTOR at a similar concentration to that used for PI3K inhibition [39]. Faced with this possible lack of LY294002 specificity, we also employed a direct inhibitor of AKT, designated AKT inhibitor I, which acts by competitively binding the AKT pleckstrin homology domain of the kinase [40]. AKT inhibitor I (10–40 μmol/l) was shown to suppress AKT phosphorylation in a dose-dependent manner, yet it exerted no effect on mTOR signaling in cells with and without activated AKT [41]. In A549 cells, AKT inhibitor I inhibited significantly acrolein-induced Bad phosphorylation (Fig. 1F and G).

To confirm further the implication of PI3K/AKT in Bad phosphorylation, we used another well-known PI3K inhibitor, Wortmannin. In contrast to LY294002 and AKT inhibitor I, Wortmannin is effective at low nanomolar doses, which suggests that it may be more specific than the other two inhibitors [40]. Wortmannin binds irreversibly to PI3K. Although Wortmannin can also have off-target effects, there appear to be effective concentrations (i.e., 100 nM) at which the inhibitor is PI3K selective [39]. Indeed, Wortmannin inhibited Bad phosphorylation in acrolein treated cells (Fig. 1F and G). Together, the findings with the three inhibirors LY294002, Wortmannn and AKT inhibitor I suggest that Bad phosphorylation was mediated by PI3K/AKT (Fig. 1).

3.1.3. Acrolein increased cIAP1/2 expression in A549 cells

The expression of cellular inhibitors of apoptosis proteins (cIAPs) is also known to be regulated by the PI3K/AKT pathway [42,43]. The cIAP proteins function as intrinsic regulators of the caspase cascade [44]. We evaluated the capacity of acrolein to regulate the expression of cIAP1/2 in A549 cells (Fig. 2A–D). Indeed, acrolein (3–14 μM) induced an increase in total cellular expression of cIAP1/2, ranging from 37 to 84% above the control level. Accordingly, the aldehyde-induced increase in cIAP1/2 expression was inhibited by LY294002, AKT inhibitor I and Wortmannin, suggesting that this response was mediated by the PI3K/AKT pathway.

Survivin, the only cIAP to associate with chromatin structures [45], plays a role in monitoring the success of chromosome replication and the suppression of caspase activity in the nucleus [44]. However, acrolein (3–14 μM) did not alter the expression of this antiapoptosis protein in A549 cells (data not shown).

Together, these findings show that exposure to low doses of acrolein (<15 μM) activated survival responses in A549 cells (Figs. 1 and 2). However, if survival responses do not succeed in counteracting a toxic insult, then the cell will be eliminated by regulated death processes such as apoptosis. We subsequently

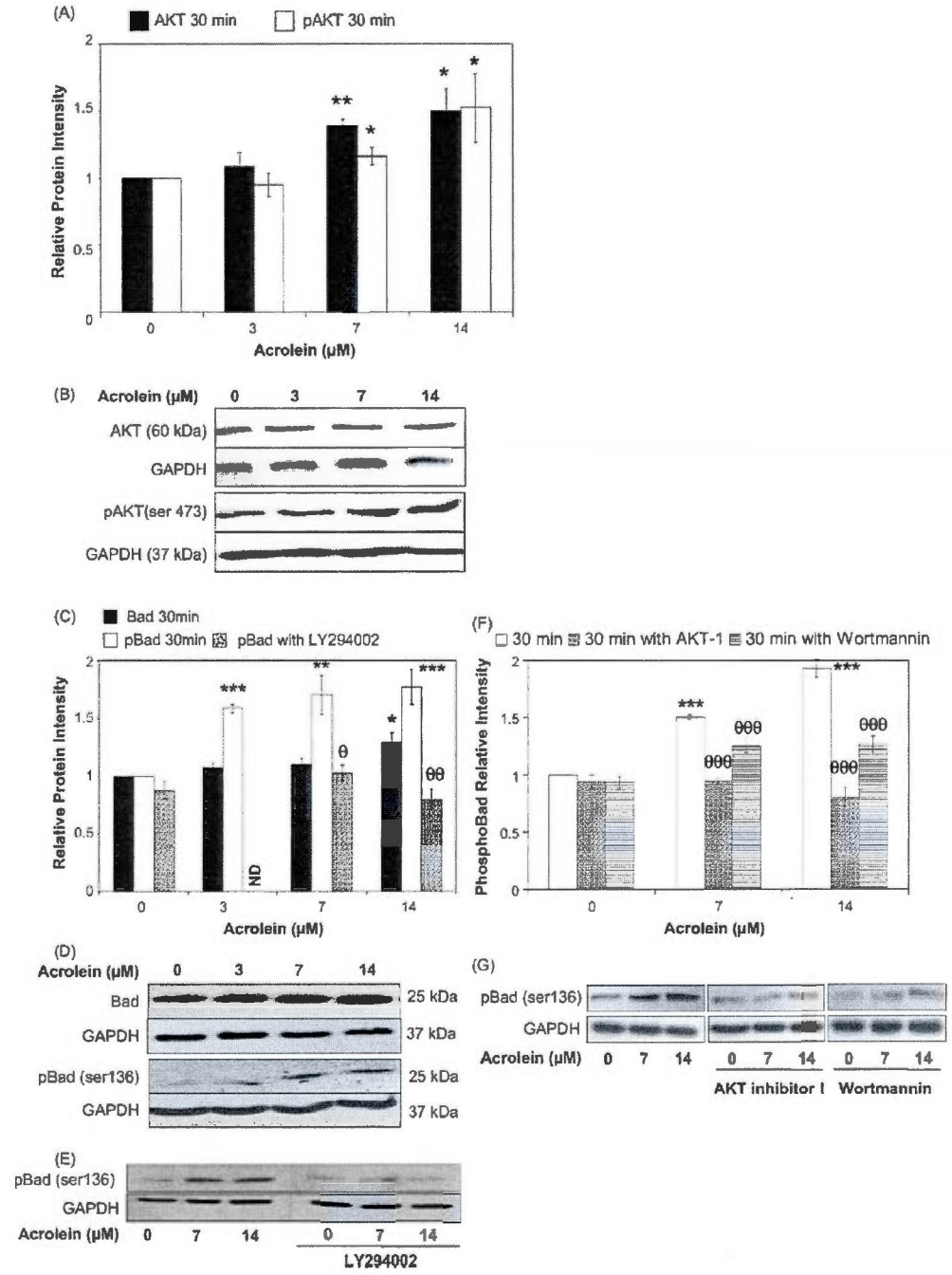

Fig. 1. Acrolein induces activation of AKT and phosphorylation of Bad. (A–G) A549 cells (10^6/ml) were incubated for 30 min at 37 °C with acrolein (0–14 μM) in α-MEM containing 10% FBS. (C and E–G) Where indicated, cells were pretreated with 25 μM of LY294002 for 1 h, or 10 μM AKT inhibitor I or 50 nM Wortmannin for 30 min, and then exposed to acrolein (0–14 μM) for 30 min. Immunodetection of AKT and PhosphoAKT (ser473) (60 kDa), and Bad and PhosphoBad (ser136) (25 kDa) in whole cell lysates was carried out by Western blotting, using GAPDH (37 kDa) as loading control. Densitometric analyses of the expression of (A) AKT and PhosphoAKT (ser473), and (C) Bad and (C and F) PhosphoBad (ser136) are relative to the untreated control. (B, D, E and G) Representative blots from three independent experiments are shown. $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P < 0.05$ (θ), $P < 0.01$ (θθ) and $P < 0.001$ (θθθ) indicates a statistically significant difference between cells exposed to acrolein, with or without LY294002, AKT inhibitor I or Wortmannin, at the same concentration of acrolein.

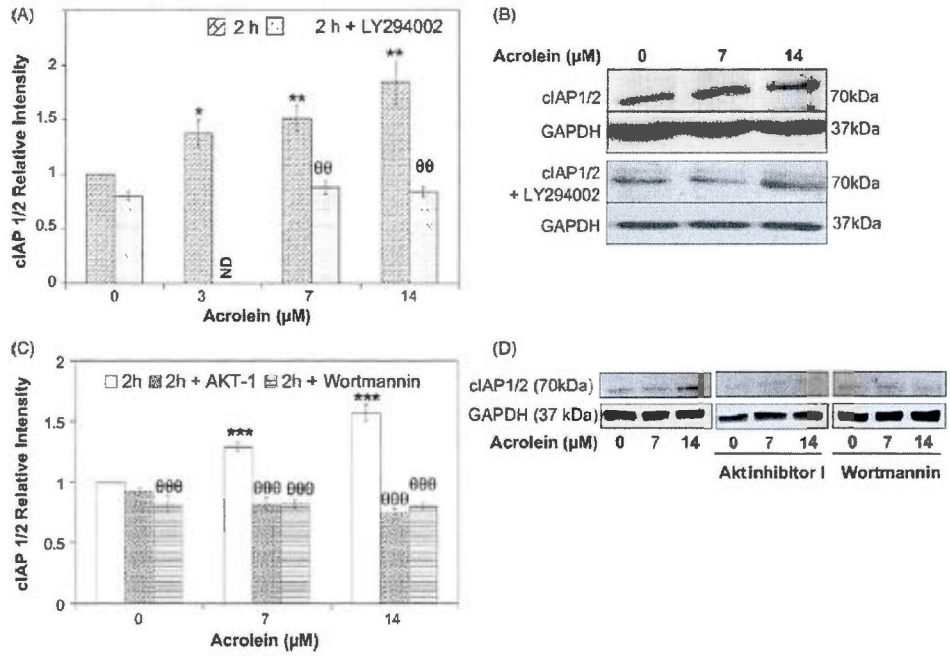

Fig. 2. Acrolein increases cIAP 1/2 expression in A549 cells. Cells (10^6/ml) were pretreated with or without 25 μM of LY294002 for 1 h, or 10 μM AKT inhibitor I or 50 nM Wortmannin for 30 min, and then exposed to acrolein (0–14 μM) for 2 h at 37 °C in α-MEM containing 10% FBS. Immunodetection of cIAP 1/2 (70 kDa) in whole cell lysates was carried out by Western blotting, using GAPDH (37 kDa) as loading control. Densitometric analyses of the expression of (A and C) cIAP 1/2 are relative to the untreated control. (B and D) Representative blots are shown from three independent experiments. $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P<0.01$ (θθ) or $P<0.001$ (θθθ) indicates a statistically significant difference between cells exposed to acrolein, with or without LY294002, AKT inhibitor I or Wortmannin, at the same concentration of acrolein.

determined the relation between the activation of antiapoptosis survival factors by acrolein and the induction of apoptosis.

3.2. Acrolein induced apoptosis through the mitochondrial pathway in A549 cells

3.2.1. Acrolein increased mitochondrial membrane potential and ROS production

The potential ability of acrolein to induce apoptosis by the mitochondrial signaling pathway was determined in A549 lung cells. We first evaluated the effect of acrolein on mitochondrial membrane potential, which is considered a critical checkpoint in the cascade of events leading to cell death. The exposure of cells to low concentrations of acrolein (10–50 μM) caused rapid (30–60 min) hyperpolarisation of the mitochondrial membrane (Fig. 3A–E). After 30 min, Rho123 fluorescence increased by about 40–60%, compared to the untreated control, for 10–50 μM acrolein (Fig. 3A). FCCP was used as a positive control and decreased membrane potential by about 60% (Fig. 3A).

Since acrolein increases mitochondrial membrane potential, we therefore investigated whether this could arise from increased ROS production. Several studies have linked mitochondrial membrane hyperpolarisation with ROS production [46–49]. Indeed, acrolein (10–50 μM) increased ROS production by about 20–46% after 30 and 60 min in A549 cells, when compared to untreated cells (Fig. 3F and G). Hydrogen peroxide was used as a positive control for ROS and increased H_2DCFDA fluorescence by a factor of about 3.2 (Fig. 3F and G). To confirm whether ROS were responsible for mitochondrial membrane hyperpolarisation, the ROS scavengers MnTBAP, sodium pyruvate, catalase and polyethylene glycol-catalase (PEG-catalase) were used (Fig. 3C and D). MnTBAP detoxifies superoxide. Catalase scavenges hydrogen peroxide at the extracellular level, while sodium pyruvate and PEG-catalase detoxify peroxide at the intracellular level [21]. Pretreatment of cells with PEG-catalase for 3 h increased cellular catalase activity by 37% (Fig. 3B). An equivalent amount of PEG alone was used as a control and did not increase catalase activity (Fig. 3B). Indeed, all of the antioxidants significantly attenuated acrolein-induced mitochondrial membrane hyperpolarisation (Fig. 3C and D), suggesting that membrane hyperpolarisation was mediated by ROS. Surprisingly, PEG alone also attenuated this effect (Fig. 3D).

3.2.2. Acrolein increased Bax levels at mitochondria

Since the mitochondrial pathway is strictly regulated by the Bcl-2 family proteins [16], we determined whether acrolein could alter the proapoptotic:antiapoptotic ratio of these proteins at the mitochondrial membrane. When, A549 cells were exposed to acrolein (3–14 μM) for 1 h, there was an increase in Bax levels at the mitochondria, relative to untreated cells (Fig. 4A and D). There was a corresponding decrease in Bax levels in the cytosolic fraction after 30 min and 1 h (data not shown). However, there was no overall change in total cellular expression of Bax and Bcl-2 proteins, or in the ratio of Bax/Bcl-2 (data not shown). The purity of cytosolic and mitochondrial fractions is shown in Fig. 4E.

3.2.3. Acrolein induced cytochrome c release and caspase activation

An important step in the mitochondrial pathway is the release of cytochrome c from mitochondria into the cytosol, where it can bind Apaf-1 [50] and amplify the apoptotic cascade. Acrolein (3–14 μM)

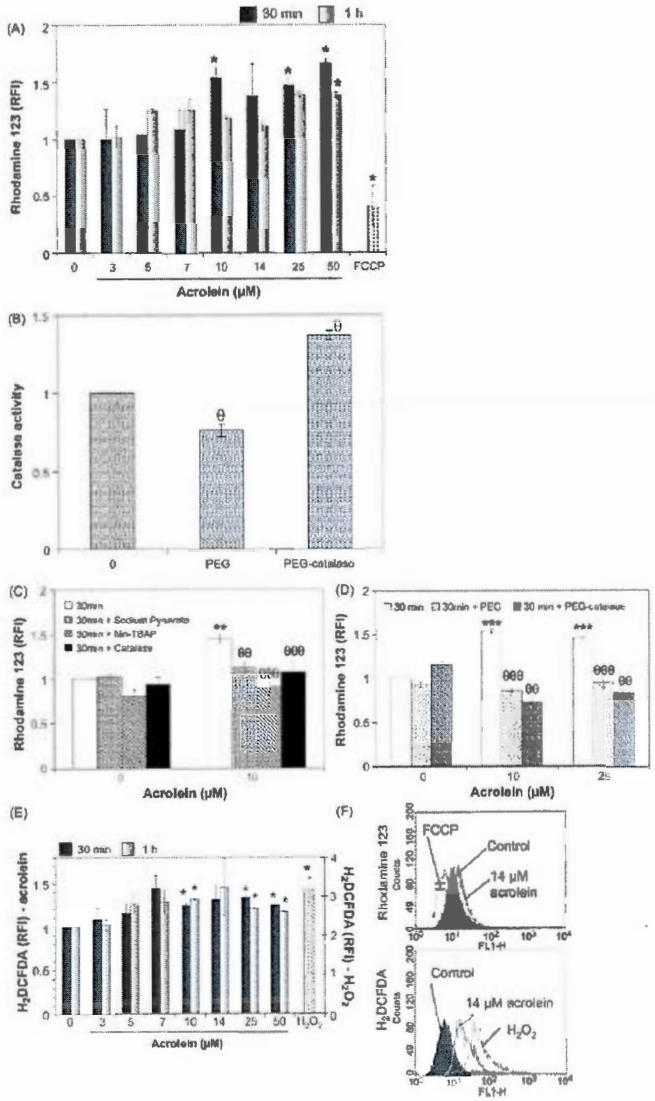

Fig. 3. Acrolein causes mitochondrial membrane hyperpolarisation and increases ROS production. (A and C–G) A549 cells (10^6/ml) were incubated with acrolein (0–50 μM) for 30 min or 1 h at 37 °C in α-MEM containing 10% FBS. Acrolein-treated cells, relative to untreated control cells, were analysed by flow cytometry for (A and E) Rho123 (2 μM), or (F and G) H_2DCFDA (10 μM) fluorescence in channel FL1 (note different vertical scales for acrolein and H_2O_2). (B) Catalase activity was determined in A549 cells (5×10^6) following incubation with 300 U/ml PEG-catalase or 2.4 mM PEG (MW 5000) for 3 h. The control value of 0.0872 ± 0.002 units (μmol of peroxide consumed/min/10^6 cells) was normalized to 1.0. (C–D) A549 cells (10^6/ml) were pretreated with or without sodium pyruvate, MnTBAP, catalase, 300 U/ml PEG-catalase or 2.4 mM PEG for 3 h, then exposed to acrolein (0–50 μM) for 30 min and analysed by flow cytometry for Rho123. (E) Histogram plots are representative results of mitochondrial membrane potential in cells exposed for 1 h to 0 μM acrolein (filled line) or 14 μM acrolein (–), or for 5 min to 5 μM FCCP (···) as positive control for mitochondrial membrane depolarisation. (G) Representative histogram plots are shown for ROS production in cells exposed for 1 h to 0 μM (filled line), 14 μM acrolein (–), and to 1 mM H_2O_2 (···) as a positive control for ROS. Data represent means and SEM of relative fluorescence intensity (RFI) from three independent experiments, normalized to controls (1.0). $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P < 0.05$ (θ), $P < 0.01$ (θθ) or $P < 0.01$ (θθθ) indicates a statistically significant difference between cells exposed to acrolein, with or without sodium pyruvate, MnTBAP, catalase, PEG MW 5000 or PEG-catalase, at the same concentration of acrolein.

caused liberation of cytochrome *c* into the cytosol after 30 min in A549 cells (Fig. 4C and D). Cytochrome *c* levels decreased by about 40% in the mitochondrial fraction, relative to untreated cells (Fig. 4B and D). The binding of cytochrome *c* to Apaf-1 leads to assembly of the apoptosome, which promotes caspase-9 activation [16]. Exposure of cells to acrolein (1–27 μM) for 30 min to 2 h increased the enzymatic activity of initiator caspase-9 (Fig. 5A).

Activation of caspase-9 then propagates the caspase cascade through activation of effector caspases [18]. To determine the downstream events from caspase-9 in the apoptotic cascade initiated

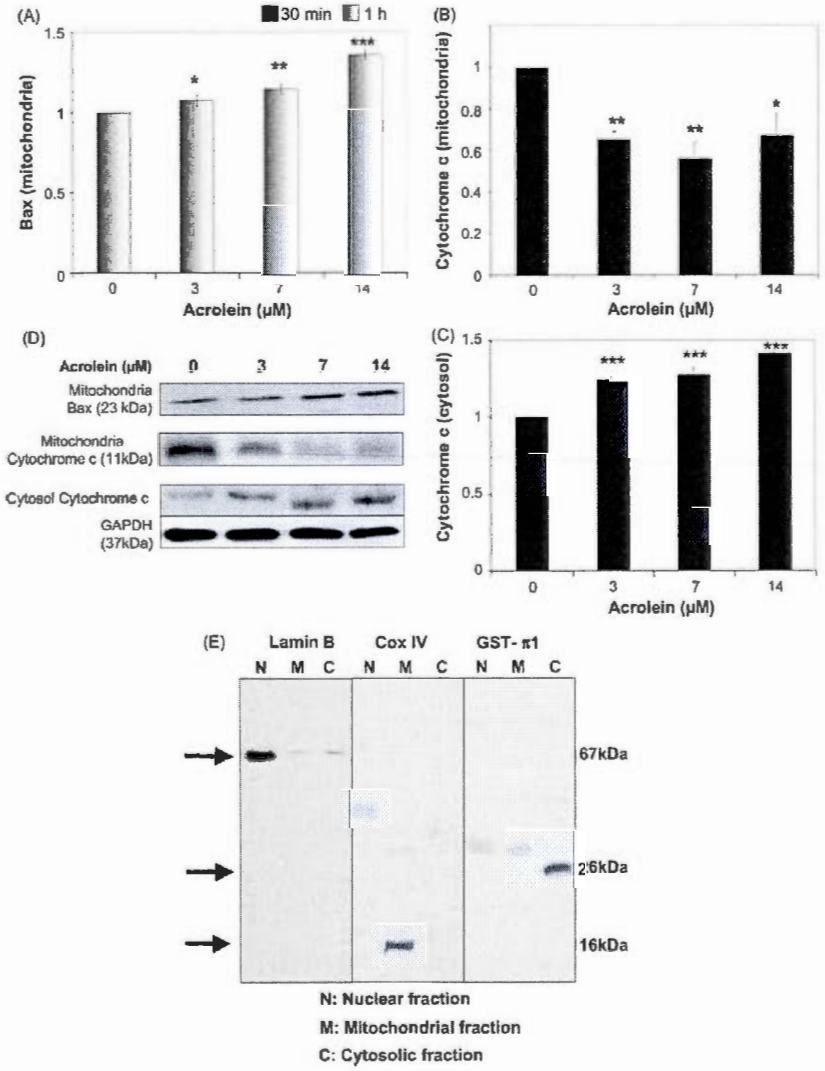

Fig. 4. Acrolein increases mitochondrial Bax levels and induces cytochrome *c* release from mitochondria. A549 cells (10^6/ml) were incubated with acrolein (0–14 μM) for 30 min or 1 h at 37 °C in α-MEM containing 10% FBS. (A and D) Levels of Bax (23 kDa) in mitochondrial fractions after 1 h were analysed by Western blotting and densitometry, relative to the untreated control (1.0). (B–D) Immunodetection of cytochrome *c* (11 kDa) in mitochondrial and cytosolic fractions after 30 min was carried out by Western blotting, using GAPDH (37 kDa) as loading control for the cytosolic fraction. Densitometric analyses of levels of (B) mitochondrial and (C) cytosolic cytochrome *c* are relative to the untreated control (1.0). (D) A representative blot is shown from three independent experiments. $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. (E) A representative blot is shown for purity of subcellular fractions, using lamin B, glutathione S-transferase (GST-π1) and cytochrome oxidase (COX IV) for nuclear, cytosolic and mitochondrial fractions, respectively.

by acrolein exposure, we looked at the enzymatic activity of effector caspases-3, -6 and -7. Caspase-3 activity increased after 1 and 2 h exposures to acrolein (1–10 μM), when compared to untreated cells (Fig. 5B). Acrolein (1–7 μM) caused a very small increase in enzymatic activity of caspase-6 after 1 and 2 h (Fig. 5D). However, there was no increase in caspase-3 or caspase-6 activity at higher doses (14–27 μM). Furthermore, acrolein (1–27 μM) also increased caspase-7 activity (Fig. 5C). To confirm the role of ROS in acrolein-induced apoptosis, cells were pretreated with PEG-catalase. Indeed, the antioxidant diminished significantly acrolein-induced caspase-3 activation (Fig. 5E).

3.2.4. *Acrolein induced AIF release from mitochondria and translocation to the nucleus*

Another factor that can be released from mitochondria into the cytosol during apoptosis is AIF, which normally resides in the intermembrane space [16]. AIF can translocate to the nucleus and contribute to DNA degradation [51], in a caspase-independent manner. We subsequently investigated whether acrolein could induce translocation of AIF to the nucleus. There was a significant increase in AIF levels in the nuclear fraction (Fig. 6A and D). Fig. 4E shows the purity of the nuclear fraction. AIF levels decreased in the mitochondrial (Fig. 6B) and cytosolic (Fig. 6C) fractions, after exposure

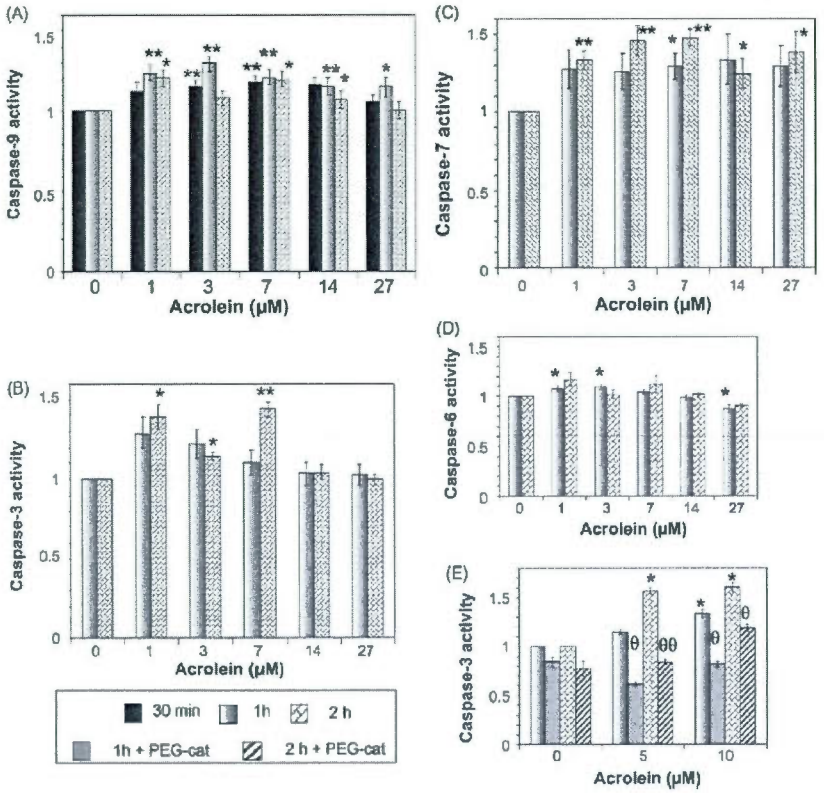

Fig. 5. Acrolein causes activation of initiator caspase-9 and effector caspases-3, -6 and -7. A549 cells (10^6/ml) were incubated with acrolein (0–27 μM) for 30 min, 1 or 2 h at 37 °C in α-MEM containing 10% FBS. Activities of (A) caspase-9, (B and E) caspase-3, (D) caspase-6 and (C) caspase-7 in cell lysates were measured using appropriate fluorescent substrates. (E) Caspase-3 activity in cells that were pretreated with or without 300 U/ml PEG-catalase for 3 h and then exposed to acrolein (0–10 μM) for 1 or 2 h. Caspase activity is shown relative to untreated controls (1.0). Data represent means and SEM from three independent experiments performed with multiple estimations per point. (A–E) $P<0.05$ (*) or $P<0.01$ (**) indicates a statistically significant difference between treatment with acrolein and the untreated control. (E) $P<0.05$ (θ) or $P<0.01$ (θθ) indicates a statistically significant difference between cells exposed to acrolein, with or without PEG-catalase, at the same concentration of acrolein.

to acrolein (3–14 μM) for 30 and 60 min. This indicates that acrolein induced AIF release from mitochondria and its translocation to the nucleus, via the cytosol.

3.2.5. Activation of the execution phase of apoptosis by acrolein

After establishing that acrolein activated the mitochondrial pathway in A549 cells, the execution phase of apoptosis was analysed at the level of cell cycle changes (Fig. 7A and B) and cleavage of PARP (Fig. 7C and D). The percentage of cells in the sub-G0/G1 population, corresponding to fragmented DNA in apoptotic cells, increased up to 22% in acrolein-treated cells, compared to untreated cells (Fig. 7A and B). The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle did not change after 24 h exposure to acrolein (1–27 μM). The majority of cells were in G0/G1 phase (~75%), whereas ~19% were in S phase and about 8% were in G2/M. Furthermore, the DNA repair protein PARP underwent cleavage and expression of the cleavage fragment (85 kDa) increased by 71% after a 2 h exposure to acrolein (25–50 μM, Fig. 7C and D). To confirm the role of ROS in acrolein-induced apoptosis, cells were pretreated with the antioxidant PEG-catalase. Indeed, PEG-catalase diminished significantly acrolein-induced cleavage of PARP (Fig. 7C and D). PEG was used as a control and did not significantly decrease PARP cleavage.

3.2.6. Acrolein induces externalization of phosphatidylserine (PS)

The externalization of phosphatidylserine (PS) is a widely used marker of apoptosis that is considered to be an early event in the execution phase. It can be revealed by Annexin V-FITC labelling of externalized PS on the plasma membrane (Fig. 8). PI was used as a counterstain for necrosis (data not shown). There was a 14% increase in Annexin V-positive cells following a 2 h exposure of A549 cells to acrolein (7–14 μM), compared to the untreated control. The role of ROS in this response was confirmed by inhibition of acrolein-induced PS externalization by PEG-catalase. Surprisingly, acrolein-induced PS externalization was diminished by PEG alone as control, although to a lesser extent than PEG-catalase. Under these conditions, levels of necrotic cells remained low (~2%) and were not affected by PEG-catalase or the general caspase inhibitor ZVAD (data not shown).

3.2.7. Induction of cytotoxicity by acrolein

Cytotoxicity of acrolein was assessed by a clonogenic cell survival assay in A549 cells. We established that concentrations of 100 μM of acrolein and higher induced cytotoxicity after 1 h (Fig. 9). A concentration of 100 μM decreased the fraction of surviving cells by 40%. Above 100 μM, there was a marked decline in cell survival. To confirm the role of ROS in acrolein-induced cytotox-

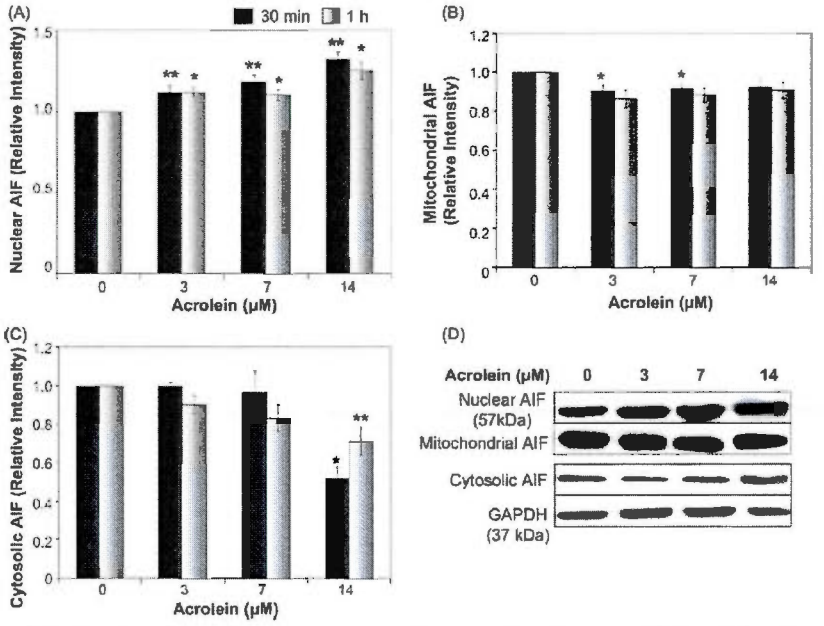

Fig. 6. Acrolein induces release of AIF from mitochondria and translocation to the nucleus. A549 cells (10^6/ml) were exposed to acrolein (0–14 μM) for 30 min or 1 h at 37 °C in α-MEM containing 10% FBS. Immunodetection of mitochondrial, cytosolic and nuclear AIF (57 kDa) was carried out by Western blotting, using GAPDH (37 kDa) as loading control for the cytosolic fraction. Densitometric analyses for the expression of AIF in (A) nuclear, (B) mitochondrial, and (C) cytosolic fractions are relative to untreated controls (1.0). (D) A representative blot from three independent experiments is shown for cells exposed to acrolein for 30 min. $P < 0.05$ (*) or $P < 0.01$ (**) indicates a statistically significant difference between treatment with acrolein and the untreated control.

icity, cells were pretreated with PEG-catalase or PEG. Indeed, the antioxidant diminished significantly acrolein-induced cytotoxicity, whereas PEG had no effect. The role of caspases in acrolein-induced cytotoxicity was verified using the caspase inhibitor Z-VAD. Acrolein-induced cytotoxicity was not affected by ZVAD (Fig. 9), indicating that loss of clonogenic cell survival is not mediated by caspases.

4. Discussion

4.1. Acrolein induced an antiapoptosis stress response in A549 lung cells

4.1.1. Activation of AKT and phosphorylation of Bad

This study shows that exposure of A549 lung cells to low doses of acrolein (<15 μM) caused induction of the antiapoptosis survival factors, AKT and IAPs. AKT promotes cell survival by exerting an antiapoptosis effect against various stimuli including growth factors, hormones and external stresses [37]. Acrolein induced rapid (30 min) activation of AKT, as well as phosphorylation of Bad at $Ser^{136}P$, in A549 cells. Acrolein also activated AKT in CHO cells [52]. Bad regulates apoptosis upstream of mitochondrial-mediated caspase-9 activation, by binding to Bcl-2 and Bcl-XL, thus neutralizing their antiapoptotic activity [53]. However, when Bad is phosphorylated at $Ser^{136}P$ by AKT activation, it is sequestered by the protein 14-3-3 for proteosomal degradation, which prevents its proapoptotic action [38].

4.1.2. Increased cIAP1/2 expression in acrolein-treated A549 cells

The antiapoptosis proteins cIAP-1 and cIAP-2 were found to directly inhibit certain caspases, in particular caspases-9, -3 and -7, by ubiquitination [44,54]. This is the first study to show that low doses (<15 μM) of acrolein induce upregulation of the cIAP1/2 proteins. Several studies demonstrated a link between cIAP and AKT. The enhanced transcription and translation of cIAP-2 was mediated by activated AKT [42]. The overexpression of AKT resulted in the upregulation of cIAP-1 expression [43]. These results suggest a pivotal role of AKT in the regulation of cell survival through the upregulation of a specific IAP. However, acrolein did not alter expression of the IAP survivin in A549 cells. Survivin is restricted to expression at the G2/M checkpoint in the cell cycle, and is the only IAP to associate with chromatin structures [45]. The inhibitory effects obtained using three different PI3K/AKT inhibitors LY294002, Wortmannin and AKT inhibitor I, suggest that this survival response could be associated with acrolein-induced AKT activation.

4.2. Acrolein increased ROS generation and caused mitochondrial apoptosis in A549 cells

Acrolein is probably the most toxic aldehyde known, yet our findings indicate that lower doses can induce an antiapoptosis survival response. The overall fate of the cell is a question of balance between the pro- and antiapoptotic signals. Often, the fate of the cell is determined by the relative speed of simultaneous survival and death processes, and by the relative expression of different antagonists of these individual pathways [55]. In this study, even though the antiapoptosis proteins were upregulated, acrolein could still activate proapoptotic signals and execute death by apoptosis in A549 cells. In fact, during apoptosis, IAPs can be degraded by certain caspases [44], which would neutralize their antiapoptosis effect. Our findings indicate that antiapoptosis processes dominate at low

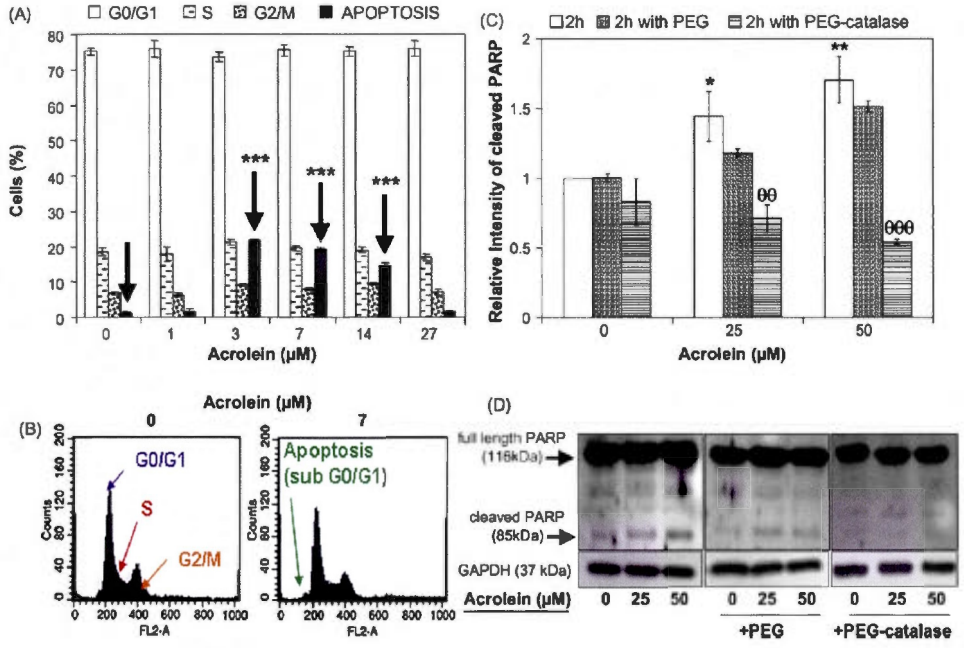

Fig. 7. Activation of the execution phase of apoptosis by acrolein. (A) A549 cells (10^6/ml) were incubated with acrolein (0–27 μM) for 24 h at 37 °C in α-MEM containing 10% FBS. Using PI as a marker for cell cycle, 10 000 cells were then analysed by flow cytometry to determine the percentage of cells in each phase. Data represent means and SEM from three independent experiments. (B) Representative histograms of flow cytometry analysis for cell cycle in channel FL2-A are shown for untreated cells and for those treated with 7 μM of acrolein. (C) Immunodetection of cleaved PARP (85 kDa) in whole cell lysates was carried out by Western blotting, using GAPDH (37 kDa) as loading control. A549 cells (10^6/ml) were pretreated with or without PEG–catalase (300 U/ml) or PEG (2.4 mM) for 3 h and then exposed to acrolein (25 or 50 μM) for 2 h in α-MEM containing 10% FBS. Densitometric analyses of the expression of cleaved PARP are relative to the untreated control (1.0). (D) A representative blot is shown from three independent experiments. $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P < 0.01$ (θθ) or $P < 0.01$ (θθθ) indicates a statistically significant difference between cells exposed to acrolein, with or without PEG–catalase or PEG, at the same concentration of acrolein.

dose (<15 μM)/shorter exposure times to acrolein, whereas propapoptotic processes dominate at higher dose (10–50 μM)/longer exposure times.

4.2.1. Acrolein caused ROS mediated mitochondrial membrane hyperpolarisation

This study establishes that acrolein induced ROS overproduction in human pulmonary A549 cells and that cell death occurred by apoptosis rather than necrosis. Acrolein was reported to stimulate ROS production and inflict mitochondrial dysfunction in PC-12 cells, but cell death occurred by necrosis [56]. In addition, acrolein increased levels of ROS production and caused DNA damage (8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) formation) in HepG2 cells [57]. Increased ROS production could arise through acrolein-induced depletion of the antioxidant glutathione [2].

Acrolein caused hyperpolarisation of the mitochondrial membrane in A549 cells. Apoptosis has been associated traditionally with loss of mitochondrial membrane potential. However, more recent studies have reported a transient increase in mitochondrial membrane potential (hyperpolarisation) during induction of apoptosis by a variety of stresses [58]. These stresses include staurosporine [59], tributyltin [60], H_2O_2, cisplatin, camptothecin, parathion, and γ-irradiation [58]. In vascular smooth muscle cells, high-glucose- and angiotensin-II-induced mitochondrial membrane hyperpolarisation, which reflects impairment of mitochondrial electron transfer and respiration, enhanced NAD(P)H oxidase activity (a major source of ROS generation) by increasing the cytosolic NADH:NAD+ ratio, which in turn induced upregulation of ROS production [48].

The mechanisms involved in membrane hyperpolarisation are currently unknown. However, this study establishes for the first time that acrolein causes ROS production, which in turn caused mitochondrial membrane hyperpolarisation. The role of ROS in acrolein-induced membrane hyperpolarisation was confirmed by the inhibitory effects of different antioxidants such as MnTBAP, sodium pyruvate, catalase and PEG-catalase in A549 cells. Surprisingly, PEG alone also inhibited acrolein-induced membrane hyperpolarisation. However, PEG is a known membrane repair agent. It was reported that PEG does not act as a ROS scavenger, but instead, it can prevent ROS damage by repairing disrupted plasma membranes and protecting mitochondria through direct interaction [61,62]. PEG treatment suppressed ROS elevation and lipid peroxidation levels in an *in vitro* spinal cord injury model. The inhibition of ROS and lipid peroxidation was attributed to PEG-mediated membrane repair. Indeed, membrane repair by compounds such as PEG could be an effective means for reducing ROS genesis [61]. So it would appear that PEG-catalase could be protective against acrolein-induced mitochondrial membrane hyperpolarisation, acting both as an antioxidant (catalase) and as a membrane protective agent (PEG).

Several studies have reported that acrolein induced mitochondrial dysfunction [56,63,64]. Acrolein (10–1000 μM, 60 min) caused inhibition of NADH- and succinate-linked mitochondrial respiration, change of mitochondrial permeability transition, and enzyme

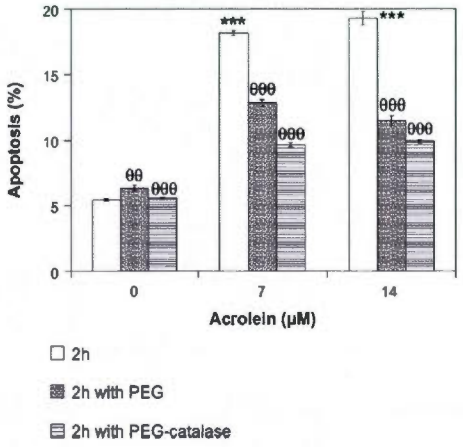

Fig. 8. Acrolein induces externalization of phosphatidylserine (PS). A549 cells (10^6/ml) were pretreated with or without PEG-catalase (300 U/ml) or PEG (2.4 mM) for 3 h, or Z-VAD (10 μM) for 1 h, and then exposed to acrolein (0–14 μM) for 2 h at 37 °C in α-MEM containing 10% FBS. Cells were stained for apoptosis with Annexin V-FITC and for necrosis with PI (data not shown). Cells (10 000) were analysed by flow cytometry to determine the percentages of Annexin V-positive and PI-positive cells. Data represent means and SEM from three independent experiments and the percentages are relative to the untreated control. $P < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P < 0.01$ (θθ), $P < 0.001$ (θθθ) indicates a statistically significant difference between cells exposed to acrolein, with or without PEG-catalase, or PEG, at the same concentration of acrolein.

inhibition of mitochondrial complex I, II, pyruvate dehydrogenase, and alpha-ketoglutarate dehydrogenase, in isolated mitochondria [64]. Acrolein (75 μM, 24 h) caused a decrease in membrane potential, and decreased activities of mitochondrial complexes I, II, and V and dehydrogenases, in a human retinal pigment epithelial (RPE) cell line [63].

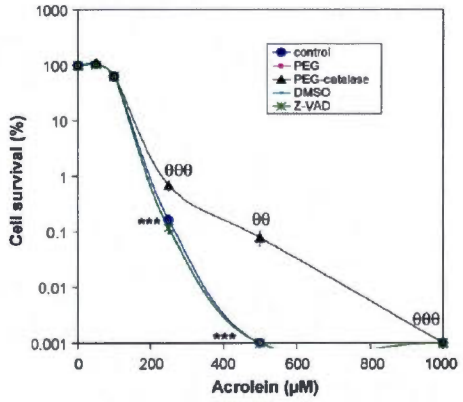

Fig. 9. Induction of cytotoxicity by acrolein. Cytotoxicity was assessed by a clonogenic cell survival assay. A549 cells (10^5/ml) were pretreated with or without PEG-catalase (300 U/ml) or PEG (2.4 mM) for 3 h, or DMSO or Z-VAD (10 μM) for 1 h, and then exposed to acrolein for 1 h at 37 °C in 1 ml of α-MEM containing 10% FBS. Acrolein was then removed, and cells were diluted and incubated in culture dishes for 10 days to allow the formation of macroscopic colonies (see Section 2). The control value represents 10^5 cells and was normalized to represent 100% cell survival. $P < 0.001$ (***) indicates a statistically significant difference between treatment with acrolein and the untreated control. $P < 0.01$ (θθ), $P < 0.001$ (θθθ) indicates a statistically significant difference between cells exposed to acrolein, with or without PEG-catalase, at the same concentration of acrolein.

4.2.2. Acrolein activated the mitochondrial pathway of apoptosis in A549 cells

The mitochondrial membrane potential has been reported to decrease, increase or not change, during cytochrome *c* release [65]. Some authors demonstrated that cytochrome c release was upstream of caspase activation and that loss of mitochondrial membrane potential was not required for its release, suggesting that depolarisation was a consequence of caspase activation and not the cause of it [49,66]. Cytochrome *c* release can also be explained by formation of a pore in the outer mitochondrial membrane, composed of tBid/Bax [16], which would not involve opening of the mitochondrial permeability transition pore or loss of mitochondrial membrane potential. Consequently, acrolein-induced activation of the mitochondrial pathway of apoptosis does not necessarily depend on opening of the mitochondrial permeability transition pore.

In most *in vitro* studies, proapoptosis protein Bax shows a different intracellular distribution before and after apoptotic stimuli [16]. BH3-only proteins such as Bid (full length and protease-cleaved, tBid, forms) have been shown to provoke conformational changes in Bax, and insertion of Bax into the outer mitochondrial membrane, which triggers cytochrome *c* release [67,68]. Acrolein (3–14 μM) stimulated Bax translocation to mitochondria in A549 cells, without changing the cellular ratio between Bax and Bcl-2 proteins, as well as liberation of cytochrome *c* into the cytosol. Our findings suggest that acrolein induces cytochrome *c* release by mechanisms which appear to involve proapoptosis Bcl-2 proteins, rather than loss of MMP.

4.2.3. Acrolein activated caspases and the execution phase of apoptosis

At the post-mitochondrial level, cytochrome *c* is an essential component of the cytosolic apoptosome complex, along with Apaf-1 and procaspase-9, which then propagate the caspase cascade [16,18]. Our results show that acrolein caused activation of initiator caspase-9 and effector caspases-3, -7 and -6. Caspase activation by acrolein appears to be cell type dependent. Acrolein also induced caspase-9 and caspase-7 activation in CHO cells [30], whereas caspase-9 inhibition occurred in murine FL5.12 proB lymphocytes [69]. Acrolein activated caspase-3 in A549 cells and in rat hippocampal neurons [70], whereas caspase-3 was inhibited in CHO cells [30], PC-12 cells [71], lymphocytes [69], and neutrophils [72]. It was suggested that the weak activation of certain caspases may arise from a partial inhibition of their active site cysteine residue through direct alkylation by acrolein [69]. It appears that acrolein can simultaneously activate and inhibit certain caspases and the final outcome (activation or inhibition) is the net result of these two processes [30].

Activation of the execution phase of apoptosis by acrolein in A549 cells was confirmed by PARP cleavage and externalization of PS after 2 h, and DNA fragmentation after 24 h. However, quenching of ROS by the antioxidant PEG-catalase inhibited acrolein-induced caspase-3 activation, PARP cleavage and PS externalization, indicating a role for ROS in these processes. PARP cleavage does not involve membrane damage, and accordingly, pretreatment of cells with PEG did not inhibit cleavage. However, PEG inhibited PS externalization, and could be acting by preventing or repairing plasma membrane damage [61]. Acrolein (25 μM, 24 h) induced apoptosis in other cell types including human alveolar macrophages [73], bronchial epithelial cells [74], and after shorter times (<50 μM, 1–2 h) in CHO cells [30]. Higher doses of acrolein (>50 μM) caused massive disruption of cellular structure and function [2,69], and cell death occurred by necrosis [30,56,71,73,74].

4.2.4. Acrolein induced AIF release from mitochondria and translocation to the nucleus

AIF normally resides in the mitochondrial intermembrane space and can act as a ROS scavenger, enabling cells to cope more effectively with oxidative stress. But once AIF is released during apoptosis, it translocates to the nucleus where it induces cell death by binding DNA, stimulating DNAse activity, triggering chromatin condensation and DNA fragmentation, in a caspase-independent manner [75]. This study shows, for the first time, that low doses (<15 μM) of acrolein induced AIF translocation from mitochondria to the nucleus in A549 cells after short exposure times (30–60 min). Thus, it appears that mitochondrial proteins released by acrolein can trigger apoptosis by both caspase-dependent and caspase-independent mechanisms.

In conclusion, the novel findings show that exposure to low doses of acrolein (<15 μM) induced a rapid (30 min) antiapoptosis cellular stress response through the activation of AKT, which led to phosphorylation of Bad and upregulation of cIAP1/2, in A549 human lung cells. Acrolein (10–50 μM, 30–60 min) triggered a rapid increase in ROS production and mitochondrial changes, such as membrane hyperpolarisation and the release of cytochrome c and AIF. However, the execution events of apoptosis, such as activation of executor caspases and PARP cleavage, occurred after longer exposure times (1–2 h) to higher doses of acrolein (10–50 μM). These findings are relevant to the toxicity of acrolein in multiple contexts, including the pharmacological action and/or side effects of the anticancer agent cyclophosphamide, which has acrolein as one of its metabolites, the regulation of cellular proliferation and tumor growth by polyamines, Alzheimer's, lung and cardiovascular diseases, as well as the toxicity of environmental exposures to low doses of acrolein. Nevertheless, further studies are necessary to increase our knowledge on the toxic mechanisms of this aldehyde, which has important repercussions for human health.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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APPENDIX B

ACROLEIN INDUCES APOPTOSIS THROUGH DEATH RECEPTOR PATHWAY IN A549 LUNG CELLS: ROLE OF P53

Acrolein induces apoptosis through the death receptor pathway in A549 lung cells: role of p53[1]

Julie Roy, Pragathi Pallepati, Ahmed Bettaieb, and Diana A. Averill-Bates

Abstract: Acrolein, a highly reactive α,β-unsaturated aldehyde, is an omnipresent environmental pollutant. Chronic and acute human exposures occur through exogenous and endogenous sources, including food, vapors of overheated cooking oil, house and forest fires, cigarette smoke, and automobile exhaust. Acrolein is a toxic byproduct of lipid peroxidation, which has been implicated in pulmonary, cardiac, and neurodegenerative diseases. This study shows that p53 is an initiating factor in acrolein-induced death receptor activation during apoptosis in A549 human lung cells. Exposure of cells to acrolein (0–50 μmol/L) mainly caused apoptosis, which was manifested by execution phase events such as condensation of nuclear chromatin, phosphatidylserine externalization, and poly(ADP-ribose) polymerase (PARP) cleavage. Levels of necrosis (~5%) were low. Acrolein triggered the death receptor pathway of apoptosis, causing elevation of Fas ligand (FasL) and translocation of adaptor protein Fas-associated death domain to the plasma membrane. Acrolein caused activation of caspase-8, caspase-2, caspase-7, and the cross-talk pathway mediated by Bid cleavage. Activation of p53 and increased expression of p53-upregulated modulator of apoptosis (PUMA) occurred in response to acrolein. FasL upregulation and caspase-8 activation were decreased by p53 inhibitor pifithrin-α and antioxidant polyethylene glycol catalase. These findings increase our knowledge about the induction of cell death pathways by acrolein, which has important implications for human health.

Key words: acrolein, apoptosis, death receptor, caspase, p53, oxidative stress, A549 cell, Fas ligand.

Résumé : L'acroléine, un aldéhyde α,β-insaturé très réactif, est un polluant omniprésent dans l'environnement. Les humains s'exposent de façon chronique et aiguë à cet agent par des sources exogènes et endogènes, par exemple l'alimentation, les vapeurs d'huile de cuisson surchauffée, les feux de forêt et d'habitation, la fumée de cigarette et les gaz d'échappement des automobiles. L'acroléine est un sous-produit toxique de la peroxydation des lipides, qui a été impliquée dans diverses maladies pulmonaires, cardiaques et neurodégénératives. La présente étude montre que p53 est un facteur déclencheur dans l'activation des récepteurs de mort induite par l'acroléine durant l'apoptose chez les cellules pulmonaires humaines A549. L'exposition des cellules à l'acroléine (0–50 μmol/L) a induit l'apoptose par des phases d'exécution comme la condensation de la chromatine nucléaire, l'externalisation de la phosphatidylsérine et le clivage de PARP. Les taux de mort par nécrose ont été faibles (~5%). L'acroléine a activé la voie apoptotique des récepteurs de mort, causant une augmentation d'expression du ligand Fas (FasL) et la translocation, à la membrane plasmatique, de la protéine adaptatrice associée au domaine de mort de Fas. L'acroléine a déclenché l'activation des caspase-8, -2 et -7, et la voie de dialogue (*cross-talk*) induite par le clivage de la protéine Bid. L'acroléine a aussi causé l'activation de p53 et l'augmentation de l'expression du gène PUMA. L'augmentation d'expression de Fas et l'activation de la caspase-8 ont été diminuées par l'inhibiteur de p53, pifithrine, et l'antioxydant, polyéthylène glycol catalase. Ces résultats améliorent nos connaissances sur l'induction des voies apoptotiques par l'acroléine, qui a des répercussions importante sur la santé humaine.

Mots-clés : acroléine, apoptose, récepteur de mort, caspase, p53, stress oxydatif, cellule A549, ligand Fas.

[Traduit par la Rédaction]

Introduction

Acrolein, an α,β-unsaturated aldehyde, is an omnipresent environmental pollutant to which humans are exposed in a wide variety of contexts (WHO 2002; USEPA 2003). It is released naturally to the atmosphere as a product of fermentation and ripening. Acrolein is generated during forest and house fires. A major source of acrolein exposure is cigarette smoke. Acrolein is found in food and drink (WHO 2002), and in vapors of overheated cooking oil, where toxic exposures have occurred (Beauchamp et al. 1985). In addition to exogenous sources of exposure, acrolein is produced in situ during the normal intermediary catabolism of various amino acids and polyamines and also when allylic alcohols or the anti-cancer drug cyclophosphamide is metabolized (Stevens and Maier 2008). Furthermore, acrolein is both a byproduct and an initiator of lipid peroxidation (Kehrer and Biswal 2000) arising from oxidative stress, which has been implicated in pulmonary, cardiac, and neurodegenerative diseases.

The level of acrolein in human serum is estimated to

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normally range as high as 50 μmol/L (Satoh et al. 1999) and to reach 80 μmol/L in fluids lining the respiratory tract of smokers (Eiserich et al. 1995). In patients with Alzheimer's, acrolein levels ranged from 50 to 100 μmol/L in some brain regions (Lovell et al. 2001). A blood level of 129 μmol/L acrolein was achieved in a patient who died of acrolein-induced acute cardiotoxicity after oral ingestion of the herbicide allyl alcohol (Toennes et al. 2002).

Acrolein is a highly selective airway toxicant (Aranyi et al. 1986) that can induce respiratory tract lesions (Beauchamp et al. 1985). Therefore, acrolein exposure has been associated with lung diseases such as asthma (Samet and Cheng 1994), chronic obstructive pulmonary disease (Hogg 2001), chronic bronchitis, emphysema (Saetta 1999), cystic fibrosis (Witschi et al. 1997), and lung carcinogenesis (Feng et al. 2006).

The mode of cell death induced by acrolein is dose- and cell type-dependent. Acrolein concentrations exceeding 50 μmol/L cause massive disruption to the structure and function of cells (Kehrer and Biswal 2000), which die by necrosis (Li et al. 1997; Tanel and Averill-Bates 2005). Necrosis is characterized by irreversible plasma membrane damage, which leads to leakage of cellular contents into the extracellular environment, often resulting in inflammatory reactions. However, at doses less than 50 μmol/L, acrolein decreased proliferation of human lung carcinoma cell lines (Rudra and Krokan 1999) and induced apoptosis in several cell types, such as alveolar macrophages (Li et al. 1997), bronchial epithelial cells (Nardini et al. 2002), and Chinese hamster ovary (CHO) cells (Tanel and Averill-Bates 2005). Acrolein inhibited apoptosis in neutrophils (Finkelstein et al. 2001) and induced oncosis and (or) necrosis in proB lymphocytes (Kern and Kehrer 2002).

A major route of human exposure to acrolein is through lung inhalation. Therefore, the A549 cell line was chosen for this study because of its human pulmonary origin. This study investigates potential initiating factors, such as p53 and reactive oxygen species (ROS), in the activation of the death receptor pathway of apoptosis by acrolein. Given the widespread exposure of humans to low doses of acrolein, this research is important for improving our knowledge about the activation of cell death pathways by acrolein.

Materials and methods

Cell culture

A549 human lung cells (ATCC No. CCL-185) were grown in a monolayer in Dulbecco's modified Eagle's medium (Invitrogen, Burlington, Canada), containing 10% fetal bovine serum (FBS) (Invitrogen Canada), 2 mmol/L L-glutamine, 3.7 g/L sodium bicarbonate, 1.0 mmol/L sodium pyruvate, and 1% penicillin (50 U/mL)–streptomycin (50 μg/mL) (Flow Laboratories, Mississauga, Canada), in tissue culture flasks (Sarstedt, Saint-Laurent, Canada). The cells were grown to near confluence, and then harvested using 0.25% (*w/v*) trypsin–0.02% (*w/v*) EDTA solution, washed by centrifugation (1000*g*, 3 min), and resuspended in minimal essential medium α (αMEM) plus 10% FBS for experimental studies (Horton et al. 1999).

Treatment with acrolein

A549 cells (10^6/mL) were exposed to acrolein (Sigma-Aldrich, Oakville, Canada) concentrations varying from 1 to 50 μmol/L (representing in vivo values) for times ranging from 30 min to 4 h, at 37 °C. Where indicated, cells were pretreated for 3 h with 10 μmol/L pifithrin-α, a p53 inhibitor (Calbiochem, La Jolla, USA) or 300 U/mL polyethylene glycol catalase (PEG catalase), an antioxidant (Sigma-Aldrich). Cells were washed by centrifugation (1000*g*, 3 min) to remove acrolein, and then analysed for apoptotic signaling.

Preparation of whole-cell lysates

After treatment with acrolein, cells were washed by centrifugation (1000*g*, 3 min) in buffer A (100 mmol/L sucrose, 1 mmol/L EGTA, 20 mmol/L 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.4) (Samali et al. 1999). The supernatant was discarded, and pelleted cells were resuspended in lysis buffer B (buffer A plus 5% Percoll, 0.01% digitonin, and a cocktail of protease inhibitors: 10 μmol/L aprotinin, 10 μmol/L pepstatin A, 10 μmol/L leupeptin, 25 μmol/L calpain inhibitor I, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF)) and incubated on ice for 1 h. Then, after a 10 min centrifugation step at 2500*g* to remove nuclei and unbroken cells, the proteins of whole-cell lysates were isolated in the supernatant for the immunodetection of p53, p53 Ser15P, p53-upregulated modulator of apoptosis (PUMA), Fas receptor (FasR), and poly(ADP-ribose) polymerase (PARP).

Preparation of subcellular fractions

Subcellular fractions were prepared as previously described (Samali et al. 1999; Gómez-Angelats and Cidlowski 2001). After treatment with acrolein, cells were washed in buffer A and then resuspended in buffer B containing 0.1 mmol/L dithiothreitol (DTT). After a 30 min incubation on ice, lysates were homogenized using a hand potter (Kontes glass, Duall 22; Fisher, Montreal, Canada), and then centrifuged at 2500*g* for 10 min. The supernatant was centrifuged again at 15000*g* for 30 min. The pellet containing the mitochondrial fraction was then resuspended in lysis buffer B for the detection of truncated Bcl-2 homology 3 (BH3)-interacting domain death agonist (tBid). The supernatant was centrifuged at 100000*g* for 1 h. The resulting supernatant was designated as the cytosolic fraction, which was used for detection of Fas-associated death domain (FADD), Bid, and receptor-interacting protein (RIP) kinase. The pellet was resuspended in lysis buffer B and designated as the microsomal fraction, which was used to detect the translocation of FADD and RIP to the plasma membrane. Purity of cytosolic, mitochondrial, and microsomal fractions was determined by Western blotting, using glutathione *S*-transferase (GST-π1), cytochrome oxidase, and calnexin, respectively (data not shown). The microsomal extracts contained plasma membranes (Gómez-Angelats and Cidlowski 2001).

Immunodetection

SDS–PAGE (10% for RIP, p53, p53 Ser15P, and PARP; 15% for PUMA, FasR, FADD, Bid, and tBid) of cellular proteins was carried out according to the methods of Laemmli (1970). Proteins (40 μg) were quantified according to Bradford (1976) and then solubilized in Laemmli sample buffer. Electrophoresis was carried out at a constant voltage

of 125 V. Cellular proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by using a MilliBlot graphite electroblotter I apparatus (Millipore, Bedford, USA) (Tanel and Averill-Bates 2005). The blots were probed with the primary antibodies anti-p53, anti-PUMA, anti-FasR, anti-FADD, anti-RIP, anti-Bid, anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA), anti-tBid, and anti-p53 Ser15P (Stressgen, San Diego, USA) in Tris-buffered saline (50 mmol/L Tris base, 150 mmol/L NaCl, and 0.1% Tween 20) (TBS-T) containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Membranes were washed and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit, or anti-goat IgG (Biosource, Camarillo, USA) diluted in TBS-T containing 5% milk powder. Proteins were detected using the ECL Plus chemiluminescence kit (PerkinElmer, Boston, USA). Protein expression was analysed with a scanning laser densitometer and expressed relative to GAPDH (Molecular Dynamics, Sunnyvale, USA).

Flow cytometric analysis of FasR and FasL expression

After treatment with acrolein, 1×10^6 cells were incubated with either 20 µg/mL fluorescein isothiocyanate (FITC)-labelled anti-FasR (Cifone et al. 1994) or 20 µg/mL phycoerythrin (PE)-labelled anti-FasL (Kayagaki et al. 1995) (Caltag Laboratories, Burlingame, USA) for 30 min at room temperature in the dark and then washed (Filion et al. 2004). Cells (10000) were analysed by flow cytometry using a FACScan (Averill-Bates et al. 2005) equipped with an argon laser (488 nm) and analysed with CellQuest software (Becton Dickinson, Oxford, UK). The extracellular expression of FasR and FasL was compared with that of untreated cells. To control for nonspecific binding, A549 cells were labelled with appropriate isotype controls for FITC and PE. No significant differences were found compared with unlabelled cells (data not shown).

For total FasL expression, cells were pretreated with or without 300 U/mL PEG catalase or 10 µmol/L pifithrin-α for 3 h and then exposed to acrolein. Cells were then fixed with 1% formaldehyde for 20 min and permeabilized with saponin buffer (20 mmol/L Hepes, 138 mmol/L KCl, 4 mmol/L $MgCl_2$, 0.2 mg/mL saponin, 1% bovine serum albumin, 1 mmol/L ATP, and 3 mmol/L phalloidin) for 1 min. Cells were washed in saponin-free buffer and labelled for 1 h at 4 °C with 20 µg/mL PE-labelled anti-FasL (Caltag Laboratories). Total expression of FasL in 10000 cells was analysed by FACScan (Vitriol et al. 2007).

Determination of caspase activity by fluorescence spectroscopy

Caspase activity was measured as described elsewhere (Hampton and Orrenius 1997), with minor modifications. After treatment with acrolein, cells were washed, resuspended, and lysed at –80 °C for 30 min (Tanel and Averill-Bates 2005). The kinetic reaction was followed for 30 min, after addition of the appropriate caspase substrates at 37 °C, using a Spectra Max Gemini spectrofluorimeter (Molecular Devices, Sunnyvale, USA). Caspase-2 activity was measured by cleavage of the fluorogenic peptide substrate Z-Val-Asp-Val-Ala-Asp-AFC. For caspase-8, the substrate was Z-Ile-Glu-Thr-Asp-AFC; for caspase-10, it was Ala-Glu-Val-Asp-AFC; and for caspase-7, it was MCA-Val-Asp-Gln-Val-Gly-Trp-Lys-(DNP)-NH_2 (Calbiochem).

Flow cytometry analysis of mitochondrial membrane potential

A549 cells (1×10^6) were exposed to acrolein or to 5 µmol/L *p*-trifluoromethoxyphenylhydrazone (FCCP: positive control) in a final volume of 1.0 mL at 37 °C. Then, 2 µmol/L rhodamine 123 (Rho123) was added for 30 min at 37 °C in the dark. Changes in fluorescence (mitochondrial membrane potential) on the FL-1 detector (green fluorescence) in 10000 cells were measured by flow cytometry and analysed using Lysis II software (Tanel and Averill-Bates 2005).

Determination of phosphatidylserine externalization by annexin V – FITC staining

After treatment with acrolein, cells (1×10^6/mL) were washed with PBS and resuspended in 1 mL of binding buffer (10 mmol/L Hepes–NaOH (pH 7.5), 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$). A volume of 500 µL of cell suspension was incubated with 5 µL of annexin V – FITC (BD Biosciences, Mississauga, Canada) and 10 µL of propidium iodide (PI) for 10 min at room temperature in the dark (van Engeland et al. 1998). Cells (10000) were then analysed by flow cytometry (Averill-Bates et al. 2005). Annexin V – FITC fluorescence was detected on FL-1, and PI fluorescence on FL-3 (red fluorescence detector). Four populations of cells were analysed: live control cells (annexin V^-/PI^-), early-stage apoptotic cells (annexin V^+/PI^-), late-stage apoptotic cells (annexin V^+/PI^+), and necrotic cells (annexin V^-/PI^+). The results are reported as the fraction of total apoptotic cells (early- and late-stage apoptosis).

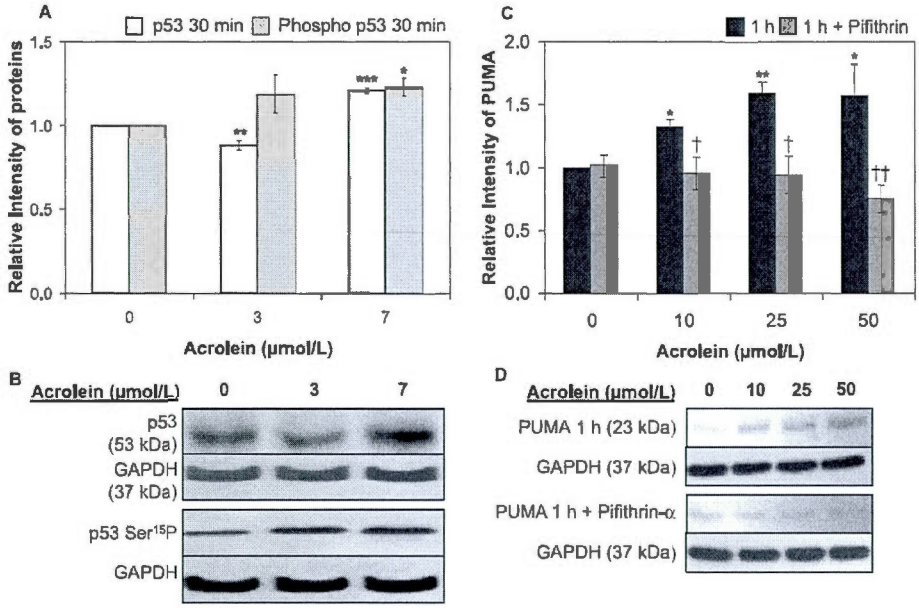
Morphological analysis of apoptosis

Cells treated with acrolein were washed, and then Hoechst 33258 (60 µg/mL) was added for 15 min to stain apoptotic cells. PI (50 µg/mL) was added to stain for necrotic cells. Images obtained by fluorescence microscopy (Carl Zeiss, Montreal, Canada) were analysed by Northern Eclipse software, and pictures were taken with a digital camera (camera 3CCD, Sony DXC-950P; Empix Imaging, Mississauga, Canada) (Averill-Bates et al. 2005). Cells were classified according to the following criteria: live cells (pale blue chromatin with organized structure), apoptotic cells (bright blue condensed or fragmented chromatin), and necrotic cells (red fluorescence). The fractions of apoptotic and necrotic cells were determined relative to total cells (obtained using bright-field illumination). A minimum of 200 cells was counted (Tanel and Averill-Bates 2005).

ROS generation

The generation of pro-oxidants was measured as described elsewhere (Denning et al. 2002), with modifications. Cells were exposed to acrolein, and then 10 µmol/L 2′7′-dihydrodichlorofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, USA) was added. H_2DCFDA produces a green fluorescence when it becomes oxidized in the presence of hydrogen peroxide. Levels of hydrogen peroxide in 10000 cells were determined by flow cytometry using the FL-1 detector.

Fig. 1. Acrolein induces activation of p53 and increases PUMA expression. A549 cells (10^6/mL) were pretreated with or without 10 μmol/L pifithrin-α for 3 h and then incubated for 30 min or 1 h at 37 °C with acrolein (0–50 μmol/L) in αMEM containing 10% FBS. Immunodetection of p53, p53 Ser15P, and PUMA in whole-cell lysates was carried out using GAPDH (37 kDa) as loading control. Densitometric analyses of the expression of (A) p53 and p53 Ser15P and (C) PUMA are relative to untreated controls (1.0). Representative blots from 3 independent experiments are shown for the expression of (C) p53 and p53 Ser15P (30 min) and for the expression of (D) PUMA (1 h). *, Significant at $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ vs. untreated control; †, $p < 0.05$ and ††, $p < 0.01$ vs. corresponding cells without pifithrin-α. PUMA, p53-upregulated modulator of apoptosis; αMEM, minimal essential medium α; FBS, fetal bovine serum.



Statistics

Data represent means ± SE from at least 3 independent experiments performed in duplicate. When not shown, error bars lie within symbols. Comparisons among multiple groups were made by one-way ANOVA, which measures the linear contrast of means. The Bonferroni–Holmes adjustment was used to control for the Family-wise error rate at a desired level (α = 5%). Software used was JMP Statistical Discovery 4.0 (SAS Institute, Cary, USA).

Results

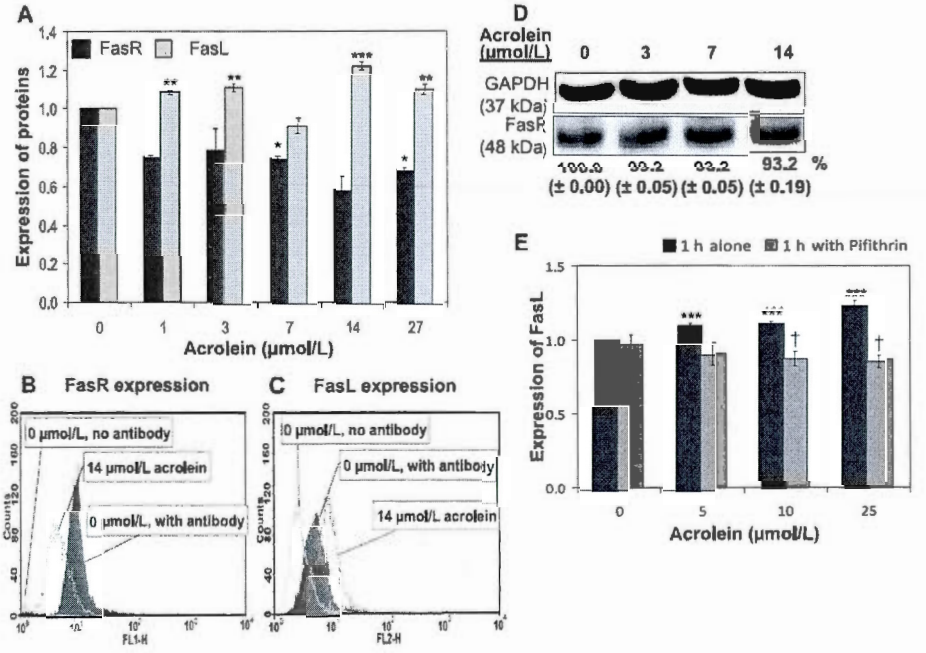
Acrolein induced activation of p53 and increased PUMA expression in A549 cells

p53 is a transcription factor as well as a tumor suppressor and is an important regulator of apoptosis. Once activated through phosphorylation or acetylation, p53 can transactivate genes involved in apoptosis, such as *Bid*, *Bax*, *Bcl-2*, *FasL*, and *PUMA* (Mendoza-Rodríguez and Cerbón 2001; Sax et al. 2002). In addition, p53 can induce apoptosis mediated by activation of caspase-2 (Maiuri et al. 2007). To determine whether acrolein can activate p53, A549 cells were exposed to low concentrations of acrolein (3–7 μmol/L) for 30 min. Acrolein (7 μmol/L) induced a significant increase (~22%) in total cellular expression of p53 and p53 Ser15P (Figs. 1A, 1B). Subsequently, the ability of acrolein to induce the expression of the p53 target PUMA was investigated. Indeed, acrolein (10–50 μmol/L) significantly increased the total cellular expression of PUMA by up to 59% (Figs. 1C, 1D). Pretreatment of cells with pifithrin-α, a reversible inhibitor of p53, significantly blocked the induction of PUMA expression. Subsequently, the role of p53 as an initiating factor in the activation of the death receptor pathway of apoptosis by acrolein was evaluated.

Role of p53 in activation of the death receptor pathway by acrolein in A549 cells

Activation of the death receptor Fas during apoptosis can be initiated by the engagement of its ligand (FasL) to its extracellular domain, which triggers the juxtaposition of its intracellular domains (Guicciardi and Gores 2003; Malhi et al. 2006). To determine the potential for toxicity of acrolein through the Fas death receptor pathway in A549 cells, the expression of FasR and FasL was evaluated by flow cytometry (Figs. 2A–2C). Compared with the untreated control, there was a significant 9%–22% increase in FasL expression

Fig. 2. Acrolein increases expression of FasL: inhibition by pifithrin-α. A549 cells (10^6/mL) were incubated for 1 h at 37 °C with acrolein (0–27 μmol/L) in αMEM containing 10% FBS. (A) Cells were analysed by flow cytometry for their external expression of FasR and FasL. Data are means ± SE from 3 independent experiments and are relative to the untreated control (1.0). The histogram plots are representative results of expression of FasR (B) or FasL (C) in control cells (0 μmol/L acrolein) with or without antibody or cells treated with 14 μmol/L acrolein. (D) Immunodetection of FasR (48 kDa) in whole-cell lysates is shown using GAPDH (37 kDa) as loading control. A representative blot for FasR is shown from 3 independent experiments. Densitometric analyses of FasR expression are relative to the control (100%). (E) Cells pretreated with or without 10 μmol/L pifithrin-α for 3 h and then analysed by flow cytometry for their total expression of FasL. *, Significant at $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ vs. untreated control; †, $p < 0.05$ vs. corresponding cells without pifithrin-α. FasL, Fas ligand; FasR, Fas receptor.



at the extracellular surface of the plasma membrane after a 1 h exposure to acrolein (1–27 μmol/L) (Figs. 2A, 2C). Under the same conditions, extracellular FasR expression decreased significantly (21%–42%) compared with untreated cells (Figs. 2A, 2B). Therefore, we further investigated whether there were changes in total expression of this receptor. Immunodetection in A549 whole-cell lysates after 1 h of exposure to acrolein (3–14 μmol/L) showed no significant changes in total expression of FasR (Fig. 2D). The decrease in extracellular FasR expression (Fig. 2A) could be explained by the binding of FasL to the extracellular surface of the receptor. The acrolein-induced increase in FasL expression was significantly inhibited by pifithrin-α, suggesting that this response was mediated by p53 (Fig. 2E).

Acrolein caused translocation of adaptor proteins FADD and RIP to the plasma membrane

Once death receptor signaling is initiated, the death domains of FasR then recruit adaptor proteins FADD and (or) RIP to the receptor complex at the cytoplasmic side of the plasma membrane (Jäättelä and Leist 2003). Indeed, acrolein (≥3 μmol/L) induced the translocation of FADD and RIP to the A549 cell membrane (Figs. 3A, 3C, 4A, and 4C), which resulted in a corresponding reduction in their respective cytosolic levels (Figs. 3B, 3C, 4B, and 4C). The maximum increases in plasma membrane translocation for FADD and RIP occurred after 1 h of exposure to acrolein, relative to untreated controls.

Acrolein induced activation of initiator caspase-8 and caspase-2 in A549 cells

The initiator procaspase-8 and procaspase-10 are recruited by adaptor proteins and accumulate at the membrane death-inducing signaling complex (DISC), resulting in their spontaneous activation by autoproteolytic cleavage, which initiates apoptotic signaling (Guicciardi and Gores 2003; Mollinedo and Gajate 2006). Caspase-2 is also an initiator caspase, but its mechanisms are not well defined. Exposure of A549 cells to acrolein (≥1 μmol/L) for 30 min to 2 h led to significant activation of initiator caspase-2 (Fig. 5A) and

Fig. 3. Acrolein causes translocation of adaptor protein FADD to the plasma membrane. A549 cells (10^6/mL) were incubated for 30 min or 1 h at 37 °C with acrolein (0–14 μmol/L). Immunodetection of FADD (30 kDa) in microsomal and cytosolic fractions was carried out using GAPDH (37 kDa) as loading control for the cytosolic fraction. Densitometric analyses of the expression of (A) membrane FADD and (B) cytosolic FADD are relative to the untreated control. (C) A representative blot for FADD expression (1 h) is shown from 3 independent experiments. *, Significant at $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ vs. untreated control. FADD, Fas-associated death domain.

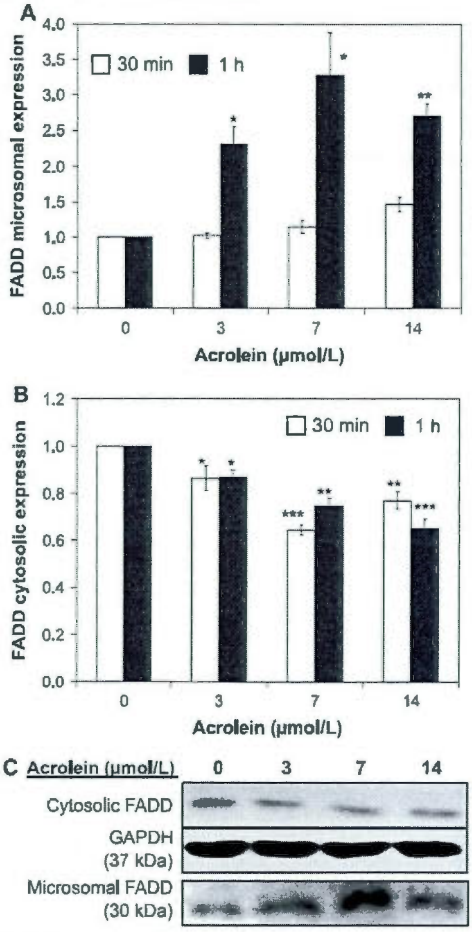
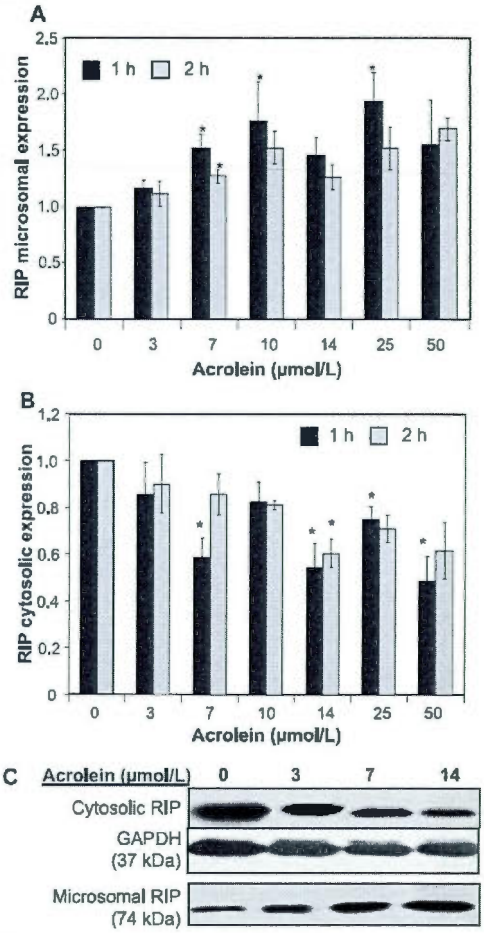


Fig. 4. Translocation of adaptor protein RIP to the plasma membrane in acrolein-treated cells. A549 cells (10^6/mL) were incubated for 1 h or 2 h at 37 °C with acrolein (0–50 μmol/L) in αMEM containing 10% FBS. Immunodetection of RIP (74 kDa) in microsomal and cytosolic fractions was carried out using GAPDH (37 kDa) as loading control for the cytosolic fraction. Densitometric analyses of the expression of (A) membrane RIP and (B) cytosolic RIP are relative to the untreated control. (C) A representative blot for RIP expression (1 h) is shown from 3 independent experiments. *, Significant at $p < 0.05$ vs. untreated control. RIP, receptor-interacting protein.

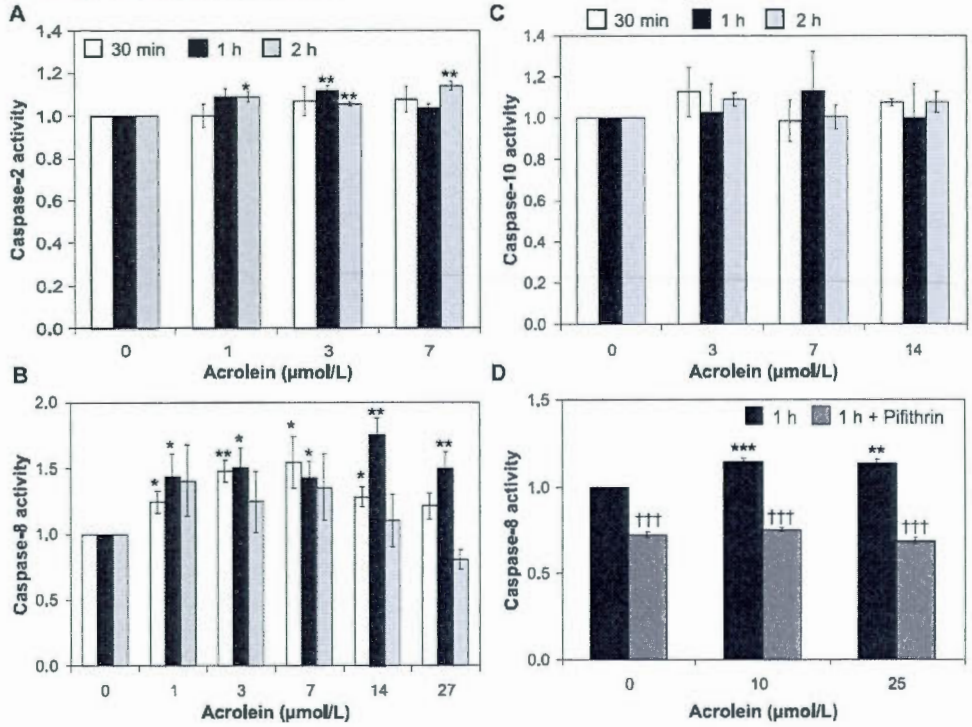


caspase-8 (Fig. 5B). Caspase-10 activity increased slightly, but this effect was not significant (Fig. 5C). The increase in enzymatic activity was more pronounced for caspase-8 than for caspase-2. (Figs. 5A, 5B). The acrolein-induced increase in caspase-8 activity was significantly inhibited by pifithrin-α, suggesting that this response was mediated by p53 (Fig. 5D).

Activation of the cross-talk pathway by acrolein

Once initiator caspase-2, caspase-8, and (or) caspase-10 are activated, they can induce a cross-talk pathway between the death receptor and mitochondrial pathways through their ability to cleave Bid (Fan et al. 2005; Lavrik et al. 2006). Exposure of A549 cells to acrolein (7–14 μmol/L) for 30 min led to a significant increase in cytosolic expression of full-length Bid by approximately 1.5-fold relative to the

Fig. 5. Acrolein induces activation of initiator caspase-2 and caspase-8. A549 cells (10^6/mL) were pretreated with or without 10 μmol/L pifithrin-α for 3 h and then incubated for 30 min, 1 h, or 2 h at 37 °C with acrolein (0–27 μmol/L). (A) Caspase-2, (C) caspase-10, and (B, D) caspase-8 activities were measured in cell lysates using the fluorescent substrates Z-VDVAD-AFC, Ac-IETD-AFC, and Z-IETD-AFC, respectively. Caspase activity was expressed relative to the untreated control. Data are means ± SE from 3 independent experiments performed with multiple estimations per point. *, Significant at $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ vs. untreated control; †††, $p < 0.001$ vs. corresponding cells without pifithrin-α.



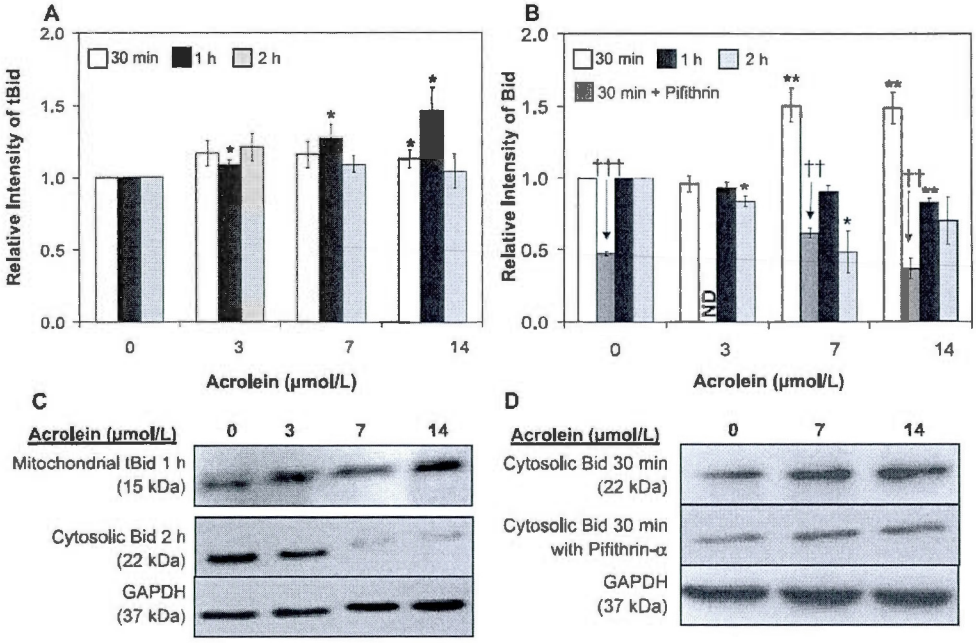
untreated control (Figs. 6B, 6D). This upregulation of Bid was significantly inhibited by pifithrin-α, implicating p53 in this effect. The increased cytosolic Bid expression observed after 30 min returned to control levels after 1 h (Fig. 6B), and there was a corresponding increase in levels of cleaved fragment tBid in the mitochondrial fraction (Figs. 6A, 6C). After 2 h, cytosolic Bid levels decreased significantly after exposure to acrolein (Fig. 6B). Once cleaved, tBid inserts itself in the mitochondrial membrane, which can lead to the activation of the mitochondrial pathway of apoptosis (Fan et al. 2005). Indeed, acrolein caused changes to mitochondria, manifested as hyperpolarization of the mitochondrial membrane (Fig. 7). This effect was significantly inhibited by pifithrin-α, suggesting that p53 was involved. FCCP was used as a positive control for membrane depolarization.

Activation of the execution phase of apoptosis by acrolein

Once activated, initiator caspases can process downstream effector caspases, which are responsible for the cleavage and degradation of many crucial cellular proteins (e.g., lamin A, actin, fodrin) and for the execution of cell death (Fan et al. 2005; Guicciardi and Gores 2003). It was reported that acrolein inhibits caspase-3 activity in CHO cells and neutrophils (Tanel and Averill-Bates 2005; Finkelstein et al. 2001). The initiator caspase-2 and caspase-8 are both known to activate the effector caspase-7 (Ho et al. 2005). Indeed, enzymatic activity of caspase-7 was increased significantly when A549 cells were exposed to acrolein (1–27 μmol/L) for 30 min to 2 h, compared with untreated controls (Fig. 8A). The maximum increase in activity was 48%, which was reached after 2 h.

Subsequently, the execution phase of apoptosis was analysed to determine the ultimate outcome of acrolein insult on the integrity of the cell. This was evaluated by PARP cleavage, by annexin V–FITC labelling of externalized phosphatidylserine (PS) on the plasma membrane (Figs. 8D–8F), and by Hoechst 33258 (blue–green fluorescence), which labels condensed nuclear chromatin (Figs. 8G, 8H) (Malhi et al. 2006). PI was used as a counterstain for necrosis. The DNA repair protein PARP underwent

Fig. 6. Cleavage of Bid to tBid by acrolein. A549 cells (10^6/mL) were pretreated with or without 10 μmol/L pifithrin-α for 3 h and then exposed to acrolein (0–14 μmol/L) for 30 min to 2 h. Immunodetection of cytosolic Bid (22 kDa) and mitochondrial tBid (15 kDa) was carried out using GAPDH (37 kDa) as loading control for the cytosolic fraction. Densitometric analyses of the expression of (A) mitochondrial tBid and (B) cytosolic Bid are relative to untreated controls. Representative blots from 3 independent experiments are shown for expression of (C) tBid (1 h) and Bid (2 h) and (D) Bid (30 min) with or without pifithrin-α. *, Significant at $p < 0.05$ and **, $p < 0.01$ vs. untreated control; ††, $p < 0.01$ and †††, $p < 0.001$ vs. corresponding cells without pifithrin-α. Bid, BH3-interacting domain death agonist; tBid, truncated Bid; ND, not determined.



cleavage, and expression of the cleavage fragment (85 kDa) increased significantly (36%) after a 2 h exposure to acrolein (25–50 μmol/L) (Figs. 8B, 8C). During the execution phase of apoptosis, caspases cause PARP-1 cleavage and inactivation. PARP cleavage has been used as a classic biochemical hallmark of apoptosis. To confirm the role of p53 in acrolein-induced PARP cleavage, cells were pretreated with pifithrin-α. Indeed, pifithrin-α significantly diminished acrolein-induced cleavage of PARP (Figs. 8B, 8C). There was a significant 3.1- to 3.7-fold increase in annexin V-positive cells after a 4 h exposure of A549 cells to acrolein (1–27 μmol/L) (Figs. 8D, 8F) compared with the untreated control (Figs. 8D, 8E). A similar 3-fold increase in apoptotic cells was obtained with Hoechst staining after exposure to 14 μmol/L acrolein compared with the control (Figs. 8G, 8H). Under these conditions, levels of necrotic cells remained low (~5%) (Figs. 8D, 8E, and 8H).

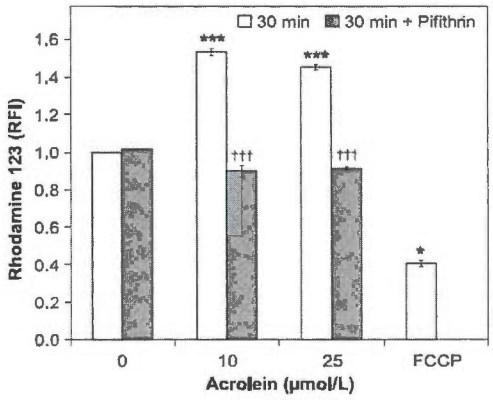
Role of ROS generation in p53 activation by acrolein

Our findings suggest that p53 plays an upstream role in activation of the death receptor pathway by acrolein in A549 cells. Subsequently, we determined whether ROS generation by acrolein is involved in the activation of p53. Acrolein (10–25 μmol/L) caused a significant 25% increase in ROS production after 1 h, manifested as an increase in H_2DCFDA fluorescence relative to the untreated control (Fig. 9A). The increase in H_2DCFDA fluorescence was inhibited by PEG catalase, a well-known ROS scavenger that detoxifies hydrogen peroxide (data not shown). Pretreatment of A549 cells with 300 U/mL PEG catalase for 3 h increased catalase activity by 37% (data not shown). Indeed, acrolein-induced p53 activation was significantly inhibited by PEG catalase (Figs. 9B, 9C), which suggests that ROS play a role in p53 activation in A549 cells. Furthermore, acrolein-induced ROS production was significantly inhibited by pifithrin-α, indicating a role for p53 in ROS generation.

Role of ROS in acrolein-induced activation of the death receptor pathway

The subsequent step was to determine whether acrolein-induced ROS can activate the death receptor pathway in A549 cells. Accordingly, acrolein-induced upregulation of FasL (Fig. 10A), caspase-8 activation (Fig. 10B), and mitochondrial membrane hyperpolarization (Fig. 10C) were all attenuated significantly by PEG catalase. Together, our findings suggest that p53 activation and ROS generation are

Fig. 7. Acrolein causes mitochondrial membrane hyperpolarization: inhibition by pifithrin-α. A549 cells (10^6/mL) were pretreated with or without 10 μmol/L pifithrin-α for 3 h and then exposed to acrolein (0–25 μmol/L) for 30 min. Acrolein-treated cells, relative to untreated control cells, were analysed by flow cytometry for rhodamine 123 (2 μmol/L) fluorescence in channel FL-1. FCCP (5 μmol/L) was added for 5 min to cells not exposed to acrolein as a positive control for mitochondrial membrane depolarization. Data are means ± SE of RFI from 3 independent experiments, normalized to controls. *, Significant at $p < 0.05$ and ***, $p < 0.001$ vs. untreated control; †††, $p < 0.01$ vs. corresponding cells without pifithrin-α. FCCP, *p*-trifluoromethoxyphenylhydrazone; RFI, relative fluorescence intensity.



interconnected and that both are involved as initiating factors in acrolein-induced death receptor activation in A549 cells.

Discussion

Upstream events involved in death receptor activation by acrolein in A549 cells

Acrolein induced activation of p53 and increased PUMA expression

The initiating factors that trigger apoptosis when cells are exposed to the toxic lipid peroxidation byproduct acrolein are not well understood. Many environmental stimuli, including ROS and chemical DNA-damaging agents, can upregulate and (or) activate p53 through phosphorylation (Xu 2003; Mendoza-Rodríguez and Cerbón 2001; Sax et al. 2002). Acrolein is known to cause DNA damage (Stevens and Maier 2008), and at low doses (3–7 μmol/L), it indeed induced rapid (30 min) upregulation and activation of p53 in A549 cells. This is the first study to show that acrolein induces p53-dependent upregulation of PUMA, a BH3-only Bcl-2 family protein and an essential mediator of DNA damage-induced apoptosis. The post-translational modification of p53 at Ser15P also occurred in CHO cells that were exposed to acrolein (50 μmol/L) for 1 h (Tanel and Averill-Bates, 2007*b*). In A549 cells, benzo[*a*]pyrene-induced p53 DNA binding and activity were inhibited by acrolein (50 fmol/cell, 30 min) (Biswal et al. 2003). However, acrolein alone did not affect levels of p53 or Ser15P-p53 (Biswal et al. 2003). This difference from our study could be explained by their use of a different incubation medium, serum-free Dulbecco's modified Eagle's medium (DMEM), whereas our study used serum-containing αMEM. Given that acrolein can react with nucleophilic substances, cellular responses to acrolein are highly dependent on culture media composition and the presence and (or) concentration of serum (Thompson and Burcham 2008). In our study, A549 cells were cultured and then exposed to acrolein in serum-containing media to avoid imposing the stress of protein withdrawal on cells, which causes severe cellular damage and necrotic processes detected as PI uptake (data not shown). However, a limitation of the current study is that 10% FBS is not physiological.

Role of p53 as an initiating factor in death receptor activation by acrolein

To advance knowledge about the mechanisms involved in acrolein-induced apoptosis, we evaluated whether the tumor suppressor p53 is an upstream trigger of death receptor activation. The novel finding in this study is that FasL upregulation and caspase-8 activation were decreased by pifithrin-α, suggesting that p53 is an initiating factor in acrolein-induced death receptor activation during apoptosis in A549 cells (Fig. 11). Indeed, p53 is known to mediate the activation of genes such as *Fas* and *FasL* during apoptosis (Mendoza-Rodríguez and Cerbón 2001; Sax et al. 2002). Moreover, the activation of execution phase events of apoptosis by acrolein, such as PARP cleavage, was also dependent on p53.

Role of ROS in p53-mediated death receptor activation by acrolein

Subsequently, we determined whether p53 is activated by ROS. Indeed, our findings show that low apoptosis-inducing doses of acrolein (10–25 μmol/L) induced ROS accumulation and ROS-dependent activation of p53 in A549 cells. Acrolein (1–100 μmol/L, 24 h) was reported to cause ROS accumulation in PC-12 cells; however, cell death occurred by necrosis (Luo et al. 2005). Increased ROS production could arise through acrolein-induced depletion of the antioxidants glutathione (Kehrer and Biswal 2000) and thioredoxin (Yang et al. 2004). In A549 cells, the antioxidant PEG catalase inhibited acrolein-induced upregulation of FasL protein, caspase-8 activation, and mitochondrial membrane hyperpolarization. This suggests that in addition to p53, ROS are an upstream initiating factor in acrolein-induced death receptor activation during apoptosis (Fig. 11). ROS were shown to induce FasL expression (Wang et al. 2008) and can regulate the activation of caspase-8 during apoptosis (Perez-Cruz et al. 2007).

The novel findings in this study are that both p53 and ROS are initiating factors in acrolein-mediated death receptor activation (Fig. 11). ROS generation was inhibited by pifithrin-α, and p53 activation was inhibited by catalase, suggesting that acrolein-induced ROS generation and p53 activation are linked. Previous reports indicate that ROS can act both upstream and downstream of p53 activation (McNeill-Blue et al. 2006; Xu 2003). Furthermore, it appears that levels of p53 can control redox levels within cells

Fig. 8. Acrolein activates the execution phase of apoptosis. A549 cells (10^6/mL) were incubated for 30 min, 1 h, 2 h, or 4 h at 37 °C with acrolein (0–27 μmol/L) in αMEM containing 10% FBS. (A) Caspase-7 activity was measured in cell lysates using the fluorescent substrate MCA-VDQVDGWK(DNP)-NH_2 and was expressed relative to the untreated control. Data are means ± SE from 3 independent experiments performed with multiple estimations per point. (B) A549 cells (10^6/mL) were pretreated with or without pifithrin-α and then exposed to acrolein (25 or 50 μmol/L) for 2 h in αMEM containing 10% FBS. Immunodetection of cleaved PARP (85 kDa) in whole-cell lysates was carried out by Western blotting, using GAPDH (37 kDa) as loading control. Densitometric analyses of the expression of cleaved PARP are relative to the untreated control. (C) A representative blot is shown from 3 independent experiments. (D) A549 cells were stained for apoptosis with annexin V – FITC and for necrosis with PI. Cells (10 000) were analysed by flow cytometry to determine the percentages of annexin V-positive and PI-positive cells. Representative dot blots are for annexin V- and PI-stained cells after exposure to 0 μmol/L (E) and 14 μmol/L acrolein (F) for 4 h. Data are means ± SE from 3 independent experiments and the percentages are relative to the untreated control. Morphological analysis of apoptosis is shown in (G) untreated A549 cells and (H) cells exposed to 14 μmol/L acrolein for 4 h. Cells were stained with Hoechst 33258 and PI and were visualized by fluorescence microscopy (magnification × 320). Photographs are representative of 5 independent experiments.*, Significant at $p < 0.05$ and **, $p < 0.01$ vs. corresponding untreated control; †, $p < 0.05$ and ††, $p < 0.01$ vs. corresponding cells without pifithrin-α. PARP, poly(ADP-ribose) polymerase; FITC, fluorescein isothiocyanate; PI, propidium iodide.

(for review, see Groeger et al. 2009). Thus, low levels of p53 can suppress ROS within cells, while higher levels of p53 can promote ROS accumulation. Inhibition of acrolein-induced ROS generation by pifithrin-α would be consistent with this. In LoVo and HCT116 cancer cells, there appeared to be a feedback signaling loop involving p21/ROS/p53 in apoptotic responses (Inoue et al. 2009). However, the complex relationship between p53 and ROS remains a matter of debate.

Downstream events in acrolein-induced death receptor activation in A549 cells

Acrolein induced translocation of adaptor proteins FADD and RIP to the plasma membrane

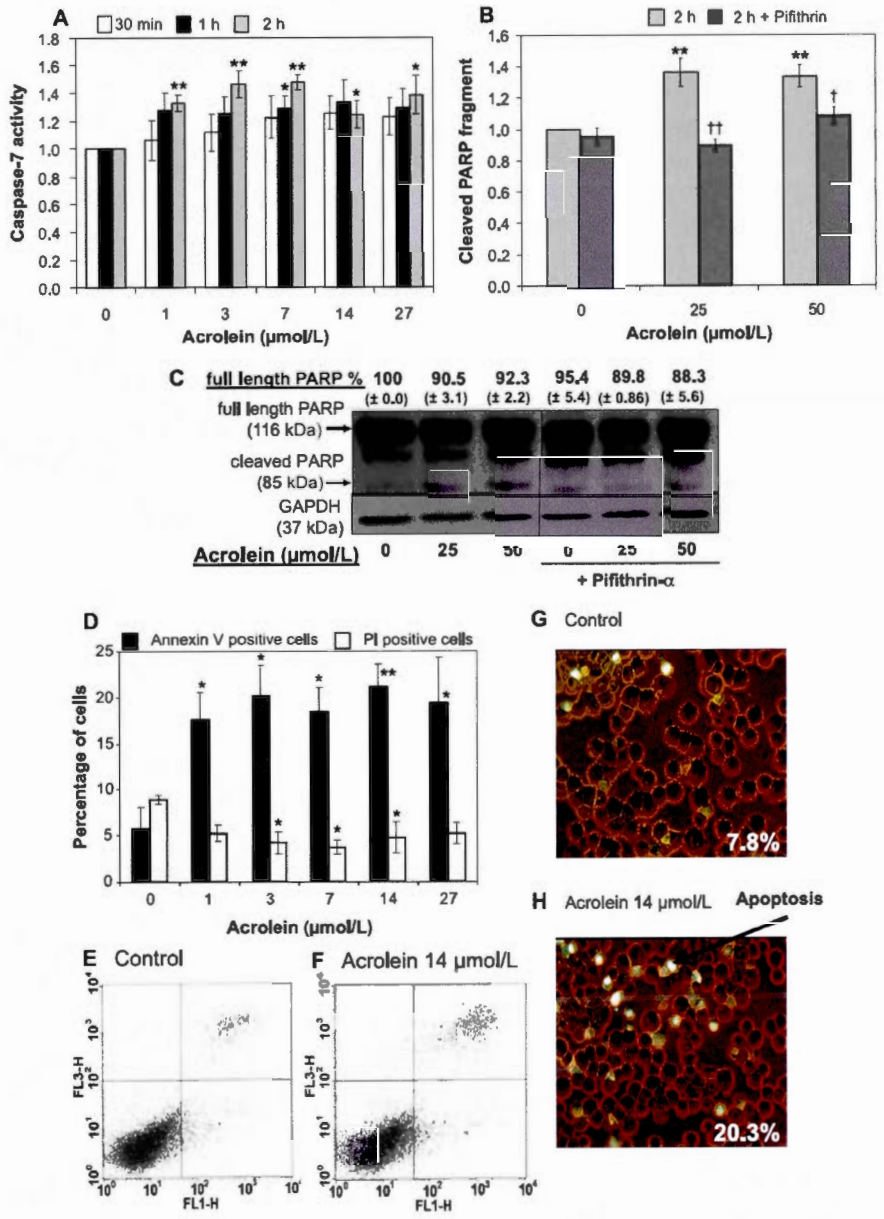
Exposure of A549 cells to acrolein led to a decrease in the expression of FasR at the extracellular surface of the plasma membrane, while total cellular content of FasR remained stable. These results suggest that increased FasL expression at the extracellular membrane encumbers FasR by specific binding, thereby preventing its detection by the FITC-labelled anti-FasR antibody. The decrease in FasR at the extracellular membrane could also be explained by internalization of the FasL–FasR cluster after receptor activation (Henkler et al. 2005). Acrolein caused Fas death receptor activation in hamster cells, which was manifested by FADD translocation, caspase-8 activation, and Bid cleavage (Tanel and Averill-Bates, 2007*a*). However, the upstream mechanisms involved in death receptor activation were not investigated.

The complexity of death receptor-induced cell survival, apoptosis, and necrosis signaling networks now exceeds, by far, that of the simple linear pathway originally suggested by the discovery of the receptor-triggered caspase cascade (Jäättelä and Leist 2003). It is well established that death receptor-mediated cell death can take the form of either apoptosis or necrosis (Laster et al. 1988; Holler et al. 2000). The adapter proteins FADD and RIP appear to be key determinants of whether death receptors trigger the apoptotic or necrotic pathway (Festjens et al. 2006). FADD is usually associated with apoptosis, whereas RIP appears to be a central initiator of necrosis. However, RIP can play a dual role and is able to initiate pro-survival as well as death-inducing pathways, although these opposing functions are not well understood (Festjens et al. 2006). In A549 cells, the concentrations (3–50 μmol/L) of acrolein that caused membrane recruitment of FADD and RIP induced morphological changes associated with apoptosis rather than necrosis. Under these conditions, RIP appears to play a role as a death inducer, giving rise to apoptosis. Given the low level of necrosis (~5%) in A549 cells, these findings indicate that death receptor activation triggered apoptosis rather than the necrosis signaling network.

Acrolein activated initiator caspase-8 and caspase-2 and the cross-talk pathway: role of p53

Caspase-8 becomes activated after recruitment to FADD at the membrane receptor and is considered to be an important initiator caspase in death receptor-mediated apoptosis (Curtin and Cotter 2003). Acrolein (1–27 μmol/L) caused p53-dependent caspase-8 activation in A549 cells. In hamster cells, low doses of acrolein (10–50 μmol/L) induced caspase-8 activation and apoptosis (Tanel and Averill-Bates, 2007*a*). Lower doses (1–10 μmol/L) of acrolein caused caspase-8 cleavage in Jurkat T (type II) cells, whereas higher doses (>10 μmol/L) were inhibitory in both Jurkat and B lymphoblastoid SKW6.4 (type I) cells (Hristova et al. 2007). However, exposure to acrolein (5–40 μmol/L) for 12 and 24 h inhibited caspase-8 activity in murine FL5.12 proB lymphocytes, and cell death occurred by oncosis and (or) necrosis (Kern and Kehrer 2002). The mode of acrolein-induced cell death is cell type-dependent.

Caspase-2 was also activated by low concentrations of acrolein (1–7 μmol/L) in A549 cells. The activation of caspase-2 by acrolein has not been reported previously. Compared with caspase-8, the molecular mechanisms of caspase-2 activation are not well defined and, in fact, are controversial. It was reported that DNA damage can activate caspase-2 in a multinuclear complex (PIDDosome) that involves the p53-induced protein with a death domain (PIDD) and the RIP-associated protein with a death domain (RAIDD) (Maiuri et al. 2007; Tinel et al. 2007). Others have reported that the death domain of RIP can bind to the Fas receptor and recruit RAIDD and caspase-2 to Fas receptor



aggregates (Curtin and Cotter 2003). In several cell lines, procaspase-2 was found at the Fas DISC and was activated on Fas stimulation. At the same time, it was reported that procaspase-2 could not initiate cell death in the absence of procaspase-8, which suggests that caspase-2 is activated at the DISC, but does not necessarily prime Fas-induced apoptosis (Lavrik et al. 2006).

There was rapid (30 min) p53-mediated upregulation of Bid

Fig. 9. Role of ROS generation in p53 activation by acrolein. A549 cells (10^6/mL) were pretreated with or without 300 U/mL PEG catalase or 10 μmol/L pifithrin-α for 3 h and then exposed to acrolein (0–25 μmol/L) for 30 min or 1 h. (A) Levels of ROS in 10 000 cells were analysed for H_2DCFDA fluorescence by flow cytometry. (B) Immunodetection of p53 Ser15P in whole-cell lysates was carried out using GAPDH (37 kDa) as loading control. Densitometric analyses of the expression of p53 Ser15P are relative to untreated controls. (C) Representative blots from 3 independent experiments are shown for expression of p53 Ser15P (30 min) with or without PEG catalase. **, Significant at $p < 0.01$ and ***, $p < 0.001$ vs. untreated control; †, $p < 0.05$ and †††, $p < 0.001$ vs. cells without PEG catalase or pifithrin-α at the same concentration of acrolein. ROS, reactive oxygen species; PEG, polyethylene glycol.

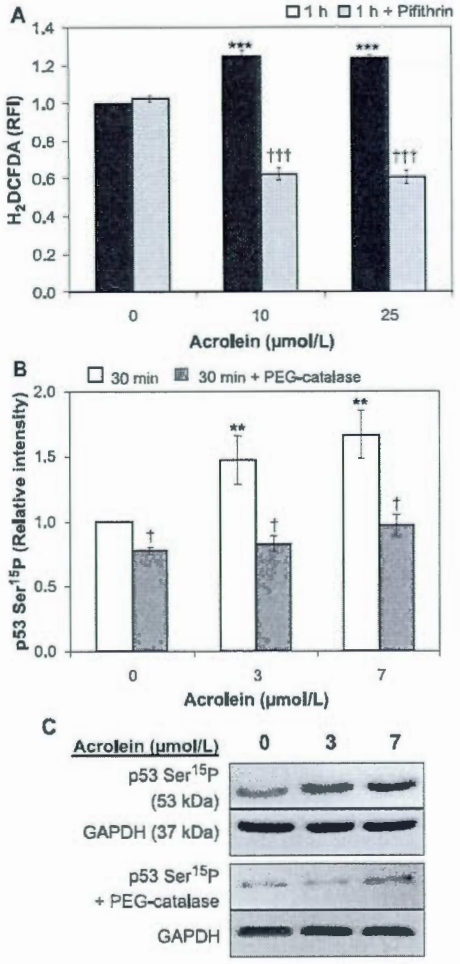


Fig. 10. Role of ROS production by acrolein in death receptor activation. A549 cells (10^6/mL) were pretreated with or without 300 U/mL PEG catalase for 3 h and then exposed to acrolein (0–50 μmol/L) for 30 min, 1 h, or 2 h. (A) Total expression of FasL, relative to untreated control cells, was analysed by flow cytometry. (B) Caspase-8 activity was measured in cell lysates using fluorescent substrate Z-IETD-AFC. Data are relative to the untreated controls and are means ± SE from 3 independent experiments performed with multiple estimations per point. (C) Mitochondrial membrane potential in 10 000 cells was analysed for rhodamine 123 fluorescence by flow cytometry. *, Significant at $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ vs. corresponding untreated control; †, $p < 0.05$, ††, $p < 0.01$, and †††, $p < 0.001$ vs. corresponding cells without PEG catalase.

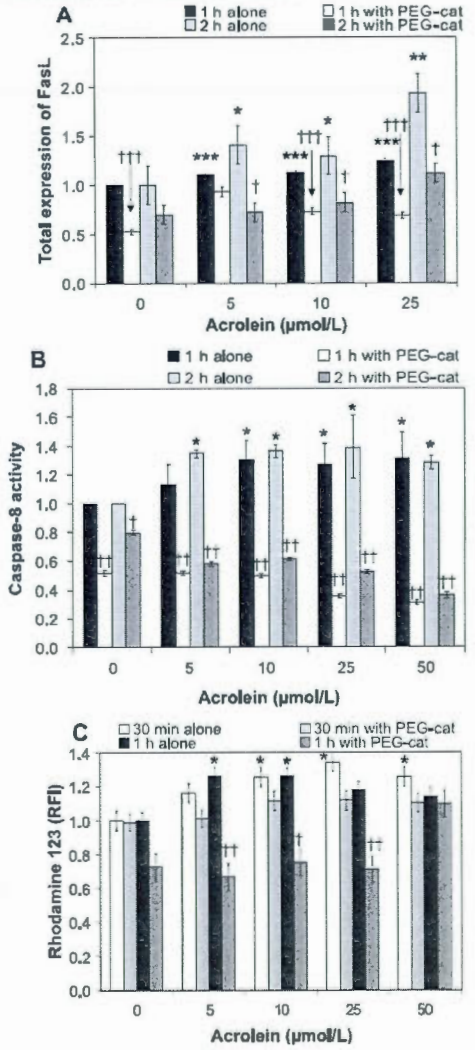
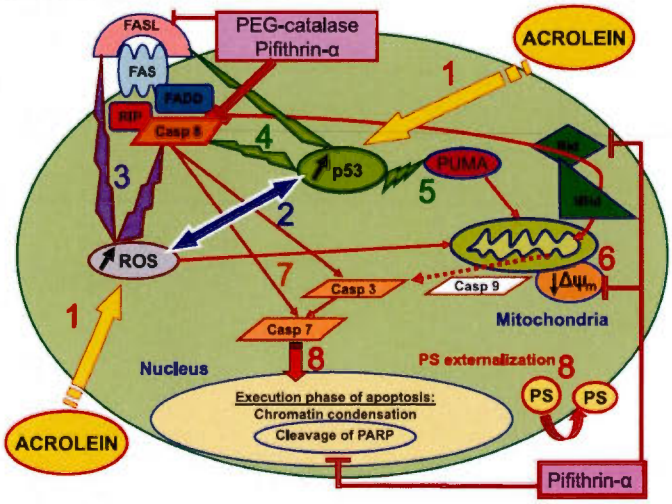


Fig. 11. Schematic diagram summarizing the pathways initiated by acrolein exposure. (1) Acrolein exposure increases levels of ROS and activates p53 in A549 lung cells. (2) ROS and p53 are both upstream factors in acrolein-induced apoptosis. Increased ROS levels are dependent on p53, and p53 activation is dependent on ROS. (3) Acrolein induces binding of FasL and FasR, thus triggering the death receptor pathway, which involves translocation of adaptor proteins FADD and RIP to the plasma membrane. ROS are involved in increased expression of FasL and caspase-8 activation, since these events are inhibited by PEG catalase. p53 activation leads to (4) caspase-8 activation and increased expression of FasL and (5) PUMA, since these events are inhibited by pifithrin-α. (6) Acrolein activates the cross-talk pathway involving caspase-8 mediated cleavage of Bid and translocation of tBid to mitochondria. Bid cleavage and hyperpolarization of the mitochondrial membrane are downstream events from p53 activation. ROS also contribute to mitochondrial membrane hyperpolarization. (7) Acrolein causes activation of caspase-9 and caspase-7. (8) Caspase-7, an effector caspase involved in the execution phase of apoptosis, is able to cleave PARP and cause chromatin condensation. Phosphatidylserine (PS) externalisation is also involved in the execution phase. PARP cleavage is dependent on p53, since it is inhibited by pifithrin-α.



expression after exposure to low doses of acrolein (7–10 μmol/L) in A549 cells. p53 can mediate apoptosis by upregulating proapoptotic genes, such as *Bid* (Sax et al. 2002). In addition to an initial upregulation of Bid in A549 cells, acrolein caused Bid cleavage to tBid, which accumulated at the mitochondrial membrane. Therefore, cytosolic levels of Bid appear to represent a balance between induction by p53 and decreased levels due to cleavage.

The molecule Bid is a well-known cleavage substrate of caspase-8, but it is also a substrate of caspase-2 (Guo et al. 2002). Therefore, both caspase-2 and caspase-8 can trigger Bid-mediated activation of the mitochondrial pathway of apoptosis (Lavrik et al. 2006). Our findings show that acrolein causes mitochondrial changes (membrane hyperpolarization) in A549 cells, which arise from death receptor signaling through the cross-talk pathway, mediated by cleavage of Bid, involving caspase-2 and caspase-8. Inhibition by pifithrin-α suggests that p53 is an upstream factor in these events (Fig. 11).

Activation of the execution phase of apoptosis by acrolein in A549 cells

Acrolein induced execution phase events of apoptosis in A549 cells, such as caspase-7 activation, PS externalization, PARP cleavage, and nuclear chromatin condensation. PARP cleavage was inhibited by pifithrin-α, confirming that execution phase events are also dependent on p53. Caspase-7 was activated by acrolein in hamster cells (Tanel and Averill-Bates 2005), is able to cleave many protein substrates that are common to caspase-3, and can induce chromatin condensation (Degterev et al. 2003). Acrolein stimulated execution phase events, such as DNA fragmentation, chromatin condensation, and PS externalization during apoptosis in isolated human alveolar macrophages (Li et al. 1997), hamster cells (Tanel and Averill-Bates 2005), and HBE1 human lung epithelial cells (Nardini et al. 2002).

Conclusions

The novel contribution to knowledge about acrolein toxicity is that p53 and ROS are upstream factors involved in activation of the death receptor pathway of apoptosis by low concentrations of acrolein (<50 μmol/L) in A549 human pulmonary cells. These findings are relevant to the toxicity of acrolein in many contexts, including the pharmacological action and (or) side effects of the anticancer agent cyclophosphamide, which has acrolein as one of its metabolites, and the regulation of cell proliferation and tumor growth by

polyamines. Acrolein is of interest from a public health perspective, since long-term exposure to chronic, low doses of this reactive aldehyde occur from both exogenous and endogenous sources.

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Abbreviations

Bax	Bcl-associated X protein
Bcl-2	B-cell lymphoma 2
Bid	BH3-interacting domain death agonist
DISC	death-inducing signaling complex
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
FACS	fluorescence-activated cell sorter
FADD	Fas-associated death domain
FasR	Fas receptor
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
αMEM	minimal essential medium alpha
MOPS	3-(*N*-morpholino)propanesulfonic acid
PARP	poly(ADP-ribose) polymerase
PE	phycoerythrin
PEG	polyethylene glycol
PI	propidium iodide
PIDD	p53-induced protein with a death domain
PS	phosphatidylserine
PUMA	p53-upregulated modulator of apoptosis
PVDF	polyvinylidene difluoride
RAIDD	RIP-associated ICH-1/Ced-3 homologous protein with a death domain
RIP	receptor-interacting protein
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
tBid	truncated Bid

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